Escherichia coli 'TatExpress' strains export several g/L human growth hormone to the periplasm by the Tat pathway

Isabel Guerrero-Montero[1†], Kirsty L. Richards[1†], Chillel Jawara[1], Douglas F. Browning[2], Amber R. Peswani[1], Mickael Labrit[3], Matthew Allen[3], Cedric Aubry[3], Emma Davé[3], David P. Humphreys[3], Stephen J. W. Busby[2] and Colin Robinson[1]*

[1] School of Biosciences, University of Kent, Ingram Building, Canterbury CT2 7NJ, UK

[2] Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK

[3] Discovery Biology, UCB Celltech, 216 Bath Road, Slough SL1 4EN, UK

[†] These authors contributed equally to this work.

* For correspondence:

Colin Robinson : Tel: +44 (0)1227- 823443. Email: C.Robinson-504@kent.ac.uk

Running title: High-level export of human growth hormone by the *E. coli* Tat pathway

Abstract

Escherichia coli is a heavily used platform for the production of biotherapeutic and other high-value proteins, and a favoured strategy is to export the protein of interest to the periplasm in order to simplify downstream processing and facilitate disulphide bond formation. The Sec pathway is the standard means of transporting the target protein but it is unable to transport complex or rapidly folding proteins because the Sec system can only transport proteins in an unfolded state. The Tat system also operates to transport proteins to the periplasm, and it has significant potential as an alternative means of recombinant protein production because it transports fully folded proteins. Here, we have tested the Tat system's full potential for the production of biotherapeutics for the first time using fed-batch fermentation. We expressed human growth hormone (hGH) with a Tat signal peptide in *E. coli* W3110 'TatExpress' strains that contain elevated levels of the Tat apparatus. This construct contained 4 amino acids from TorA at the hGH N-terminus as well as the initiation methionine from hGH, which is removed *in vivo*. We show that the protein is efficiently exported to the periplasm during extended fed-batch fermentation, to the extent that it is by far the most abundant protein in the periplasm. The protein was shown to be homogeneous, disulphide bonded and active. The bioassay showed that the yields of purified periplasmic hGH are 5.4 g/L culture whereas an ELISA gave a figure of 2.39 g/L. Separate analysis of a TorA-hGH construct lacking any additional amino acids likewise showed efficient export to the periplasm, although yields were approximately 2-fold lower.

Key words

Biopharmaceuticals, recombinant protein, E. coli, Tat system, protein secretion

1.0 Introduction

Many high-value proteins are produced in *E. coli,* and a favoured strategy is to export the protein to the periplasm, usually by the well-characterised 'Sec' pathway (Walsh, 2014). Several strategies have been used, including expression of soluble proteins in the cytoplasm, expression in the form of insoluble inclusion bodies, or export to the periplasm. The latter approach is particularly favoured for the production of proteins that contain disulphide bonds, since the periplasm is the only oxidising compartment in wild type cells (Pooley *et al.*, 1996). Downstream processing is also simplified due to the lowered levels of cytoplasmic contaminants, including DNA (Balasundaram *et al.* (2009). Export of proteins to the periplasm is usually achieved by targeting via the well-characterised Sec pathway, whereby a cleavable Sec-specific signal peptide is present at the N-terminus of the target protein. However, the Sec pathway cannot transport some heterologous proteins and attention has focused on the alternative Tat pathway, which transports fully folded proteins by a completely different pathway (reviewed by Natale *et al.*, 2008). Initial studies showed that Tat could export a model protein, GFP, at high levels and that the cells were robust under fermentation conditions (Matos *et al.*, 2012). This work was followed by additional studies that showed Tat to be capable of exporting several biotherapeutics including human growth hormone, single chain antibody fragments and interferons (DeLisa *et al.*, 2003; Alanen *et al.*, 2015; Tullman-Ercek *et al.,* 2007; Browning *et al.*, 2017; Matos *et al.*, 2014).

Human Growth Hormone (hGH) is a 22 kDa protein consisting of 191 amino acids (Pooley *et al.,* 1996) used to treat burn injuries, wound healing, hypopituitarism and obesity (Isaksson *et al.,* 1985) since it is safe to use even in high doses (Van, 1998). Since hGH only has 2

disulphide bonds (Cys53-Cys165 and Cys182-Cys189) (Ultsch *et al.,* 1994) and no glycosylation, most of its large-scale production has used *E. coli* platforms, although a wide range of eukaryotic host systems have been tested (e.g. Hahm and Chung, 2001; Ecamilla-Trevino *et al.,* 2000). The highest recorded yield is 5 g of recombinant hGH per litre of milk in transgenic cows (Salamone *et al.,* 2006).

A major difficulty in producing hGH in *E. coli* is its aggregation into insoluble bodies that complicate the production process. Until recently, most commercial hGH has been obtained from insoluble bodies that have been solubilized, with the hGH refolded into its active form (Olsen *et al.,* 1981; Patra *et al.,* 2000). Recent, novel approaches have managed to produce high quantities of cytosolic hGH in fed-batch fermentation, with reported yields of up to 678 mg/L protein (Song *et al.,* 2017).

Although studies exporting hGH to the periplasm have reported relatively low yields (Sockolosky and Szoka, 2013), this is an attractive option due to the ease of downstream processing. In recent studies, we have shown that the Tat system can export hGH with high efficiency and that it is disulphide-bonded (Alanen *et al.,* 2015). Out study concluded that, although the protein was presumably synthesised and exported in the reduced state, the periplasmic protein was fully disuphide bonded by the normal DSB disulphide bonding system. We have also reported that a new series of engineered *E. coli* strains, which overexpress the Tat system components (termed TatExpress), demonstrate even higher export efficiencies (Browning *et al.* 2017). These strains bear a modified chromosomal *tatABC* operon, the expression of which is induced by IPTG, so that the Tat system is over-expressed at the same time as the IPTG-induced target protein. However, the activity of the hGH was not assayed and our studies (Alanen *et al.,* 2015; Browning *et al.,* 2017) used laboratory shake flask systems

that bear little resemblance to industrial production processes. Here, we have assessed the feasibility of this system for industrial production using TatExpress *E. coli* strains under fed-batch fermentation conditions. We report that the system is able to produce purified, disulphide bonded, active hGH at levels that are calculated to be between 2 and 5 g/L culture.

2.0 Materials and Methods

Bacterial strains and plasmids

Strains and plasmids are summarised in Table 1. *E. coli* strain W3110 was used, together with W3110 TatExpress which was previously described in Browning *et al.,* 2017 alongside the plasmid pKRK7 (pEXT22 expressing TorA-hGH-6His). pKRK38 was derived from pKRK7 to eliminate the TorA signal peptide that precedes the hGH. pKRK38 was made by PCR amplification using pKRK7 as a template and primers Mature_hGH_F (5'-ATGTTCCCAACCATTCCCTTATCCA-3') and Mature_hGH_R (5'-CATACATGTTCCTCTGTGGTAGGGT-3') as forward and reverse primers. PCR solutions, enzymes and reaction conditions were as described in Guerrero-Montero *et al.* (in press).

A additional TorA-hGH construct was made removing the 4 initial amino acids of mature TorA and the N-terminal methionine of hGH. The construct was made by Gibson Assembly in which the hGH-His6 was amplified from plasmid pKRK7 (pEXT22, TorA-hGH-His6) and inserted into a pEXTII backbone in which the TorA signal sequence had already been cloned. Colonies were screened colony PCR and confirmation of the intended deletion was provided by sequencing from Eurofins Genomics.

Primer name	Sequence
hGH_del.FOR	AACGCCGCGACGTGCGACTGCGTTCCCAACCATTCCCTTATCCAGGC

hGH_del.REV	TTAATGGTGATGGTGATGGTGGAAGCCACAGCTGCCCTCC
TorA_del.FOR	GTGGAGGGCAGCTGTGGCTTCCACCATCACCATCACCATTAATAAGGATCTATATGACTAG
TorA_del.REV	ATAAGGGAATGGTTGGGAACGCAGTCGCACGTCGCGG

Fed-batch fermentation

Initial cultures were grown in 50 mL of 2xP media (16g/L of Bacto-tryptone/peptone, 10g/L of Yeast extract, 10 g/L NaCl) in 250 mL shake flasks for 6 h at 37°C, 200 rpm with 1:1000 antibiotic (5 µL, 1 M kanamycin). 1 mL of culture was transferred to 200 mL of SM6 defined media (Humphreys *et al*., 1998) and grown aerobically overnight at 30 °C, 200 rpm in shake flasks with 1:1000 antibiotic (5 µL, 1 M kanamycin). On the following day an equivalent of 300 OD_{600} was used to inoculate fresh defined SM6 media to a final volume of 500 mL in Infors Multifors 1.5L fermenters (Infors UK Ltd., Reigate, UK). The pH was kept at 7 using 25% (v/v) ammonia solution and 25% (v/v) sulphuric acid. Dissolved oxygen tension (DOT) was kept at 30% using gas blending with 100% oxygen where necessary and the culture was maintained at 30°C until both stirrer and airflow was maximal and then dropped to 25°C. Supplementation of $MgSO_4$ occurred when the OD_{600} reached 38-42 (8 mL/L of 1M $MgSO_4$) and of Na_2HPO_4 when the OD_{600} reached 54-58 (5 mL/L of 232.8 g/L Na_2HPO_4) and when the OD_{600} reached 66-77 (7 mL/L of 232.8 g/L Na_2HPO_4). A glycerol feed containing 80% w/w glycerol was started at OD_{600} 70, with a continuous feed of 0.01 ml/min. Induction with 9 ml/L of IPTG at a concentration of 4.31 g/L occurred at OD_{600} 75.

Fractionation

Cytoplasm, membrane and periplasm fractions were prepared by an osmotic-shock method. Cells were centrifuged at 3000 x g, 4°C, for 10 min and resuspended in 500 µL of Buffer 1 (100 mM Tris-acetate pH 8.2, 500 mM sucrose, 5 mM EDTA pH 8.0) and 500 µL dH_2O before addition of 40 µL hen egg white lysozyme (1 mg/mL) and incubation on ice for 5 min. 20 µL $MgSO_4$ (1M) was added and the suspension was centrifuged at 20,000 x g, 4 ° C, 2 min. The supernatant containing the periplasmic fraction was removed and the pellet washed once in 750 µL Buffer 2 (50 mM Tris-acetate pH 8.2, 250 mM sucrose, 10 mM $MgSO_4$). The cell pellet was resuspended in 750 µL Buffer 3 (50 mM Tris-acetate pH 8.2, 2.5 mM EDTA pH 8.0) and sonicated for 6 × 10 s, amplitude 8 µm to disrupt membranes (Soniprep 150plus, Sanyo Gallenkamp, Loughborough, UK). The suspension was centrifuged at 202,000 x g, 4 °C, 30 min to sediment the insoluble fraction. 500 µL of the supernatant was removed (designated cytoplasmic fraction). The remainder of the supernatant was discarded and the pellet resuspended in 500 µL Buffer 3 and designated membrane (or insoluble) fraction.

Protein purification

For purification of 6x Histidine-tagged (C-term) proteins by Nickel IMAC, 10 mL of the culture post induction was taken, centrifuged at 3000 rpm, 45 min, 4°C. Cell pellet was resuspended in 10 mL/g of chilled Buffer 1 without EDTA and 10 mL/g of milliQ H_2O and incubated on ice for 30 min before centrifuging at 20,000 x g, 20 min, 4°C (Beckman Avanti J- 25, JA 25.5 rotor). Supernatant was taken as the periplasmic fraction and placed into SnakeSkin® dialysis tubing (Thermo scientific) and dialysed at 4°C overnight into 50 mM sodium phosphate, 150 mM NaCl, pH 7.2. Using an ÄKTA™ pure protein purification system and a HisTrap HP histidine-tagged protein column (GE Healthcare, Buckinghamshire, UK) the protein was purified: storage solution (20% ethanol) was washed off with 10 column volumes (CV) of milliQ H_2O before adding 5 mL 0.2 M NiCl, followed by another 2CV milliQ H_2O

wash. Columns were equilibrated with 3CV equilibration buffer (50 mM sodium phosphate pH 7.2, 0.3 M NaCl) before loading Periplasmic sample and collecting Flow Through (FT). Unbound matter was removed with 6CV Wash buffer (50 mM sodium phosphate pH 7.2, 50 mM Imidazole, 0.3 M sodium chloride) and sample collected as Wash (W). Finally, the 6x Histidine-tagged protein was eluted with elution buffer with imidazole (50 mM sodium phosphate pH 7.2, 0.3 M NaCl with 250 mM Imidazole) and the peaks collected for analysis.

Protein expression analysis by Coomassie and Western blot

Protein samples were resolved by reducing SDS–PAGE and analyzed using Coomassie blue staining and Western blotting. All immunoblottingh procedures were as described in Guerrero-Montero *et al.* (in press). Periplasmic hGH activity was assayed using an hGH bioassay (PathHunter® Human Growth Hormone Bioassay Kit, Sigma) using the manufacturer's protocol. Standardized OD10 periplasmic fractions were diluted 1:500,000 using PBS and absorbance was read using a BMG Labtech Spectrostar microplate reader at 405 nm, with a reference wavelength at 490 nm. Concentrations were calculated from two independent experiments and all samples were measured in triplicate when calculating periplasmic yield. Periplasmic hGH was also assayed using an hGH ELISA kit (Roche Diagnostics, Charles Ave, Burgess Hill, West Sussex RH15 9RY) using the manufacturer's protocol. Concentrations were calculated from two independent experiments and all samples measured in triplicate, and used to calculate average periplasmic yield (in mg/L) by reference to culture OD readings at 600 nm.

Intact Protein Electrospray LC-MS

The electrospray mass spectrum was recorded on a Bruker micrOTOF-Q II mass spectrometer. An aliquot of protein in solution, corresponding to approximately 20 picomoles

of protein, was desalted on-line by reverse-phase HPLC on a Phenomenex Jupiter C4 column (5 µm, 300Å, 2.0 mm x 50 mm) running on an Agilent 1100 HPLC system at a flow rate of 0.2 ml/min using a short water, acetonitrile, 0.05% trifluoroacetic acid gradient. The eluent was monitored at 280 nm and directed into the electrospray source, operating in positive ion mode, at 4.5 kV and mass spectra recorded from 500-3000 m/z. Data were analysed and deconvoluted to give uncharged protein masses using Bruker's Compass Data Analysis software.

3.0 Results

3.1 Fed-batch fermentation of WT and TatExpress cells expressing TorA-hGH

We first expressed a construct comprising the TorA signal peptide linked to hGH (TorA-hGH) in W3110 *E. coli* TatExpress cells under fed-batch fermentation conditions as detailed in Materials and Methods. The construct was previously used in shake flask studies (Browning *et al.,* 2017) and comprises the signal peptide of *E. coli* TMAO reductase (TorA) linked to hGH via a 4-residue linker (the first 4 residues of mature TorA; AQAA). The construct encodes the initiation methione of hGH (which is removed *in vivo*) and also contains a C-terminal 6-His tag. Parallel cultures were carried out with the construct expressed in wild type (WT) W3110 cells. Figure 1 shows the growth curves from the cultures; duplicate WT and TatExpress cultures reached ODs of about 100 and the Figure shows the point at which induction of TorA-hGH and TatABC synthesis (in TatExpress) was induced using IPTG.

Samples were removed 13, 17 and 21h after induction and the cells were fractionated to generate cytoplasmic, membrane and periplasmic samples. The periplasmic samples were analysed by immunoblotting to detect the hGH and the same samples were analysed using Coomassie stained SDS PAGE gels to analyse the proteome of the periplasmic samples. Figure

2 shows the 13h and 21h samples from the two WT cultures, which indicate the presence of a clear hGH protein band in the periplasm. The Coomassie-stained gel shows the presence of a 22 kDa protein, the relative abundance of which matches the strength of the blot signals. This was confirmed to be hGH (see below). 13h, 17h and 21h samples from the two TatExpress cultures were also analysed and the data show that the hGH blot signals are significantly higher than those of the WT cultures, confirming that TatExpress cells export this construct with much higher efficiency as observed by Browning *et al.* (2017) in shake flask culture. The abundance of the 22 kDa protein is correspondingly greater in the Coomassie stained gels, providing further evidence that this is indeed hGH.

Figure 3 shows a time course analysis of the abundance of the periplasmic hGH after induction of synthesis in TatExpress cells. Samples were taken from 2 - 51 h after induction and the blot shows a steady increase in hGH level over this time period. Equal numbers of cells were used in each sample, so this reflects an increase in the amount of hGH per cell. The Coomassie gel confirms that the abundant 22 kDa protein is indeed hGH, since its abundance increases in parallel and the protein is virtually absent in the induction time point samples. Analysis of the later time points demonstrates that hGH is by far the most abundant periplasmic protein, which shows that Tat is exporting high amounts of protein.

We have carried out controls for fractionation artefacts in previous studies, and the periplasmic proteome is clearly distinct from that of the cytoplasm, suggesting that contamination of cytoplasmic proteins is minimal (e.g. Matos *et al.,* 2012). However, to confirm this point we fractionated samples at the 21 h time point and the data are shown in Figure 4. The results show that the fractions are indeed 'clean' with the periplasmic proteome clearly distinct from those of the cytoplasm and membrane fractions. To further confirm that hGH does not reach the

periplasmic fraction 'spontaneously' we expressed hGH lacking any form of signal peptide. Mature hGH was expressed in parallel with TorA-hGH and samples were fractionated after 1h and 18 h induction (Figure 5). The immunoblot shows that the bulk of hGH is in the periplasm in the TorA-hGH culture, as expected, with a minimal level of protein present in the cytoplasm at the 18h point. In contrast, hGH is found exclusively in the cytoplasm in the sample expressing mature hGH, with none detected in the periplasmic fraction. These data confirm that the presence of hGH in the periplasm of the TorA-hGH cultures is due to Tat-dependent export and not contamination by cytoplasmic fraction or spontaneous transfer across the plasma membrane.

It is notable that the total level of hGH in the culture producing mature hGH is about 6-fold lower than that in the TorA-hGH culture. This reflects a phenomenon observed previously in our shake flask studies: that hGH is subjected to rapid turnover in the cytoplasm (Alanen *et al.,* 2015). Clearly, this protein is more stable in the periplasm, and this is another advantage of using an effective protein export strategy for the production of this particular protein.

3.2 Tat-exported hGH is homogeneous and cleaved at the correct signal peptidase site

To assess the homogeneity of the exported hGH, we performed mass spectrometry analysis of the purified protein, using purified commercial hGH as a standard (provided with the Pathfinder Bioassay - see below). The exported, periplasmic hGH construct used in this study should contain additional amino acids when compared to commercial hGH, specifically the 4 amino acid linker from the mature TorA protein (TMAO reductase) at the N-terminus, the initiation methionine (cleaved from hGH *in vivo*) and the 6-His tag at the C-terminus (Figure 6). The combined molecular weight of the additional amino acids is 609.7 for the N-terminal

residues and 822.86 for the C-terminal His tag (1432.56 in total). The predicted molecular weights are 22129.05 for the commercial hGH, and 23561.5 for the exported hGH. As shown in Figure 6, mass spectrometry analysis of commercial hGH gives a single prominent peak corresponding to 22124.3 Da, which matches almost exactly with the predicted molecular mass, having only a difference of 4.75 Da, 4 of which are a consequence of the formation of 2 disulphide bonds. We purified the Tat-exported periplasmic hGH using affinity chromatography and an analysis of this protein again shows a single prominent peak, and the mass of 23555 Da is again very close to, but 6 Da smaller than the predicted mass of 23561 Da. The presence of 2 disulphide bonds again accounts for the difference. In both cases, the 1-2 Da deviation from the predicted protein masses is within the error range for the mass spectrometer.

Other smaller peaks are observed in both samples, which appear to be an artefact of the hGH mass spectrometry since they are identical in the two samples. This implies that the hGH produced in this study is (i) as homogeneous as the commercial protein (ii) processed at the correct site, and (iii) fully disulphide bonded; there is no indication of a peak corresponding to the reduced form.

To confirm that the hGH is indeed disulphide-bonded, we ran samples of the periplasmic samples from the 51 h time point shown in Figure 3 on an SDS-PAGE gel in the presence and absence of reducing agent. The oxidised, disulphide-bonded form of hGH was shown to migrate more rapidly than the reduced form (Alanen *et al.*, 2015) and Figure 7 shows the periplasmic hGH from the fed-batch fermentation likewise runs more rapidly under oxidising conditions, further confirming that the protein is disulphide-bonded. It is unclear why the

intensity of the blot signal is lower; presumably, the C-terminal His tag is less exposed in the oxidised protein and the antibodies bind less effectively.

3.3 High yields of active protein are obtained from fed-batch fermentation using TorA-hGH

To quantify the yield of periplasmic hGH and to assess its activity, we used a commercially available hGH bioassay (PathHunter® Human Growth Hormone Bioassay Kit, Sigma). This uses engineered cells in which one fragment of β -galactosidase is present on the hGH receptor and the complementary fragment is present on a phosphor-tyrosine SH2-domain-containing protein that is only able to bind the hGH receptor once it is activated. Binding of hGH to its receptor results in receptor phosphorylation by a cytosolic tyrosine kinase such as JAK1, enabling the SH2-EA fusion protein to bind the phosphorylated receptor and generate active β-galactosidase. Enzymatic activity is quantitatively measured using a chemiluminescent substrate, and the expected result is a dose-response sigmoidal function where the phosphorylation of the receptor will be dependent on the amount of hGH in the sample. A standard curve was produced with the supplied commercial hGH and all samples were done in triplicate and with dilution factors in the range of the standard curve (see Supplementary Figure 2). This assay gave a figure of 5.4 g hGH per litre of fed-batch culture for the purified periplasmic hGH shown in Supplementary Figure 1.

We also calculated hGH levels using a commercial ELISA, which uses pre-bound antibodies to hGH on the surface of the microplate modules. The sample to analyse was added in triplicate (with various dilution factors) to the wells, and the hGH present in the samples binds to the anti-hGH antibodies. Afterwards a digoxigenin-labelled antibody to hGH is added which binds

to the hGH as well. An antibody to digoxigenin conjugated to peroxidase is added and binds to the digoxigenin, and finally the peroxidase substrate ABTS is added. The peroxidase catalyses the cleavage of the substrate yielding a coloured reaction product that can be measured using a plate reader that reads at 405 and 490 nm. The absorbance is directly correlated to the level of hGH present in the sample and can be determined by comparison to the calibration curve (shown in Supplementary Figure 3). Using this ELISA the concentration of the purified periplasmic hGH was calculated to be 2.39 g/L culture. This is lower than the figure obtained using the bioassay, but since the assays are so different it is difficult to determine which is more accurate.

3.4 Export of TorA-hGH lacking the 4-residue linker between the signal peptide and hGH

The above experiments were all carried out using a construct that comprises the TorA signal peptide, 4 residues from the N-terminus of mature TorA and hGH. This construct was used in order to compare results with shake flask studies carried out using the same construct (Browning *et al.*, 2017), but we considered it important to assess the export of a 'linker-less' construct lacking any linker residues, since industrial processes are unlikely to incorporate such linkers. We therefore removed the 4 residues and also removed the hGH initiation methione so that the processed form would correspond to authentic hGH. We carried out fed-batch fermentation studies on this 'clean' construct and the periplasmic fractions are analysed in Figure 8. The immunoblot and gel data show that hGH production is induced and that high levels of hGH are exported to the periplasm over the 38 h induction period. As with the original construct, periplasmic hGH accumulates to the extent that it is a highly abundant periplasmic protein. The data thus show that removal of the 4-residue linker does not block export of hGH, and that export proceeds in the absence of the hGH initiation methionine.

Figure 9 shows a direct comparison of the export of the two hGH forms, in which fermentation samples were fractionated into cytoplasm, membrane and periplasm extracts, which were then blotted for the presence of hGH. The data show that in each case, the majority of hGH is present in the periplasm, confirming that both constructs are efficiently exported. However, the hGH immunoblot signal after export of the linker-less construct is less intense than that of the original construct containing the additional 4 residues (panel A). In addition, analysis of the stained gel in Figure 9B shows that while the linker-less periplasmic hGH is a major band, the abundance is not as striking as that of the periplasmic accumulation of hGH observed in Figures 2 and 3. We therefore believe that the linker-less hGH is exported with lowered efficiency, and quantification of the blots suggests that, on average, its export is in the region of 50% as efficient as that of the original TorA-hGH construct.

4.0 Discussion

The Tat system has been proposed to offer a viable alternative to the Sec pathway for the export of high-value proteins to the bacterial periplasm, but its potential for the production of biotherapeutic proteins has not been fully explored in previous studies. This is primarily because the majority of those studies were carried out using laboratory shake flask culture systems, whereas industrial processes almost invariably use fed-batch fermentation. Here, we have used fed-batch fermentation systems to test the robustness of the TatExpress cells and the yields of hGH that are obtained after export to the periplasm.

A major aim in this work was to test for any deleterious effects due to the increased expression of the TatABC membrane proteins in the TatExpress strain. While the TatABC proteins are expressed to a much lower extent than TorA-hGH, the increased expression of any membrane

protein can potentially lead to cell stress and lowered productivity in extended fed-batch fermentation systems. Here, we directly compared the growth characteristics of the TatExpress strain with its parental W3110 strain and we observed no significant differences. Clearly, TatExpress strains are viable production hosts.

In terms of target protein yield, we consistently observe that the cells export TorA-hGH throughout an extended induction period and it is notable that the exported hGH is by far the most abundant periplasmic protein by the end of the induction period. In control tests, mature-size hGH is not exported at all and we can conclude that TorA-hGH is being exported by the TorA signal peptide as reported in shake-flask studies (Alanen *et al.,* 2015). The yields of protein are high: the bioassay indicates a yield of over 5 g/L active purified protein while the ELISA gives a figure of 2.39 g/L. This is one of the highest yields reported and clear evidence that this platform has potential for industrial use. Nevertheless, further study would serve to illustrate the potential in more detail, and it is notable that, while a 'clean' fusion of a Tat signal peptide and hGH is efficiently exported, the presence of a short linker region appears to enhance export. An optimal signal peptide-passenger protein junction may be an important factor for efficient Tat-dependent export, as previously suggested by Tullman-Ercek *et al.,* 2007.

In summary, we have shown that a model biotherapeutic protein can be exported by the Tat system in high amounts, and that it is homogeneous, disulphide-bonded and active. It will be of interest to conduct further studies to assess the full capability of the Tat system, and in particular to explore its potential for the export of more complex proteins.

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CONFLICT OF INTEREST

The authors declare no competing financial or other interests.

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Figure Legends

Figure 1. Growth data during fed-batch fermentation of *E. coli* W3110 WT and TatExpress cells expressing TorA-hGH. Duplicate cultures were analysed of WT cells (WT1, WT2) and TatExpress (TE1, TE2), with $OD_{600\ nm}$ values shown. Fermentation was carried out at 30°C as detailed in Materials and Methods. At the indicated times, the cultures were induced with IPTG (0.1 mM) and samples were removed for analysis.

Figure 2. Tat-dependent export of TorA-hGH in W3110 wild type and TatExpress W3110 cells. Samples from duplicate fed-batch fermentation cultures of WT and TatExpress expressing TorA-hGH were removed at the indicated times post induction and fractionated to yield periplasmic samples. The periplasmic samples were analysed by Coomassie blue stained gels (A) and immunoblotting using antibodies to the His tag on hGH (B). For each lane a normalized amount of protein has been loaded, equivalent to OD_{600} 0.08 AU total cells (Coomassie blue stained gels), and OD_{600} 0.008 AU (immunoblots). Mobilites of molecular weight markers (in kDa) are shown on the left.

Figure 3. High-level export of TorA-hGH during extended fed-batch fermentation of TatExpress cells. TorA-hGH was expressed in TatExpress cells in a fed-batch fermentation system and samples were removed at the indicated times post induction (PI). The samples were fractionated to generate periplasm samples which were then analysed on Coomassie blue stained gels (A) and by immunoblotting using antibodies to the His tag on hGH (B).

Figure 4. Fractionation of samples from fed-batch fermentation of W3110 wild type and TatExpress cells expressing TorA-hGH. Samples from the 21 h time point of fed-batch

fermentation cultures of WT and TatExpress (TE) expressing TorA-hGH (shown in Figure 2) were fractionated to yield cytoplasm, membrane and periplasm samples (C, M, P). The samples were analysed by Coomassie blue stained gels (A) and immunoblotting using antibodies to the His tag on hGH (B). For each lane a normalized amount of protein has been loaded, equivalent to OD600 0.08 AU total cells (Coomassie blue stained gels), and OD600 0.008 AU (immunoblots). Mobilites of molecular weight markers (in kDa) are shown on the left

Figure 5. Mature hGH is not exported during fed-batch fermentation of TatExpress. Mature-size hGH and TorA-hGH were expressed in TatExpress cells using fed-batch fermentation systems. Samples were removed 1 h and 18 h post induction and fractionated to generate cytoplasm, membrane and periplasm samples (C, M, P) which were then analysed by immunoblotting using antibodies to the His tag on hGH.

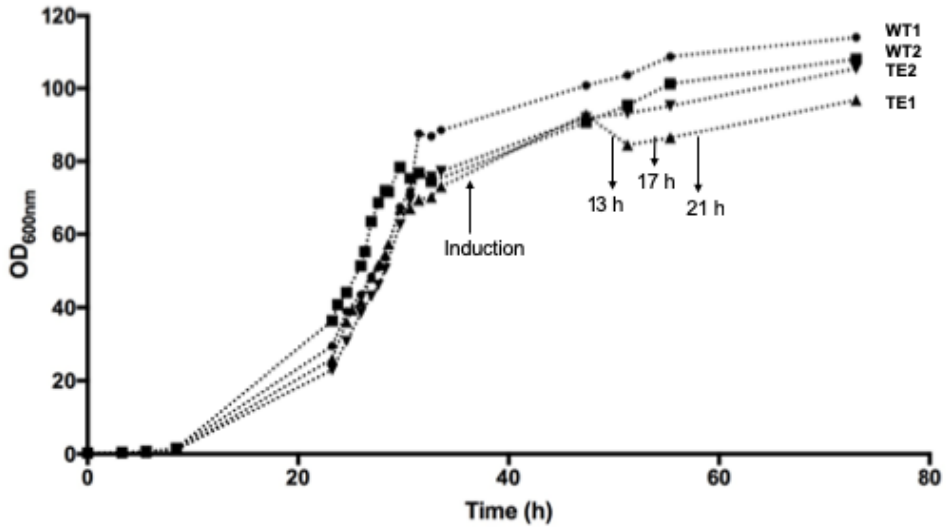
Figure 6: Exported periplasmic hGH is homogeneous and cleaved at the correct site. Periplasmic hGH was purified by affinity chromatography (see Supplementary Figure 1) and subjected to mass spectrometry analysis as detailed in Materials and Methods. Commercial hGH was analysed in an identical manner. The mass spectra for commercial hGH is shown in panel A and for periplasmic hGH in B. C: amino acid sequence of TorA-hGH-H6. The C-terminal half of the TorA signal peptide is shown, along with the first five amino acids of the mature TMAO protein and the initial methionine for the hGH protein. The signal peptidase cleavage site between ATA and AQA is denoted by ♦. The sequence for the commercial hGH is underlined, and this is followed by the 6-histidine tag.

Figure 7. Periplasmic hGH is disulphide-bonded after fed-batch fermentation. Samples of the periplasmic fraction from the 51 h time point shown in Figure 3 were run (in triplicate)

on an SDS polyacrylamide gel under standard reducing conditions ('reducing' or in the absence of reducing agent (oxidising'). The samples were immunoblotted using antibodies to the C-terminal His tag on hGH. hGH 'ox' and 'red': oxidised and reduced forms of hGH, respectively. Mobilties of molecular mass markers (in kDa) are shown on the left

Figure 8. Export of 'linkerless' TorA-hGH during fed-batch fermentation of TatExpress cells. TorA-hGH lacking a 4-residue linker and the hGH initiation methionine was expressed in TatExpress cells in a fed-batch fermentation system and samples were removed at the indicated times post induction (PI). The samples were fractionated to generate periplasm samples which were then analysed on Coomassie blue stained gels (A) and by immunoblotting using antibodies to the His tag on hGH (B). C: TorA-hGH constructs with or without the 4-residue linker were expressed in fed-batch fermentation with 40 h inductionand fractionated to generate cytoplasm, membrane and periplasm samples (C, M, P). The samples were analysed by immunoblotting using antibodies to the C-terminal His tag. hGH(ls) denotes linker-less mature hGH.

Figure 9. Comparison of export rates for hGH containing or lacking 4 additional residues at the N-terminus. TorA-hGH constructs containing 4 additional residues (as in Figures 2 and 3) or 'linker-less', lacking any additional residues between the signal peptide and mature protein (as in Figure 8; denoted hGH(ls)) were expressed in TatExpress cells as in the above Figures. After an induction period of 40 h, cells were fractionated into cytoplasm, membrane and periplasm samples (C, M, P) which were analysed by immunoblotting (A) and on Coomassie-stained SDS gels (B). Asterisks denote the mobilities of the mature hGH proteins.



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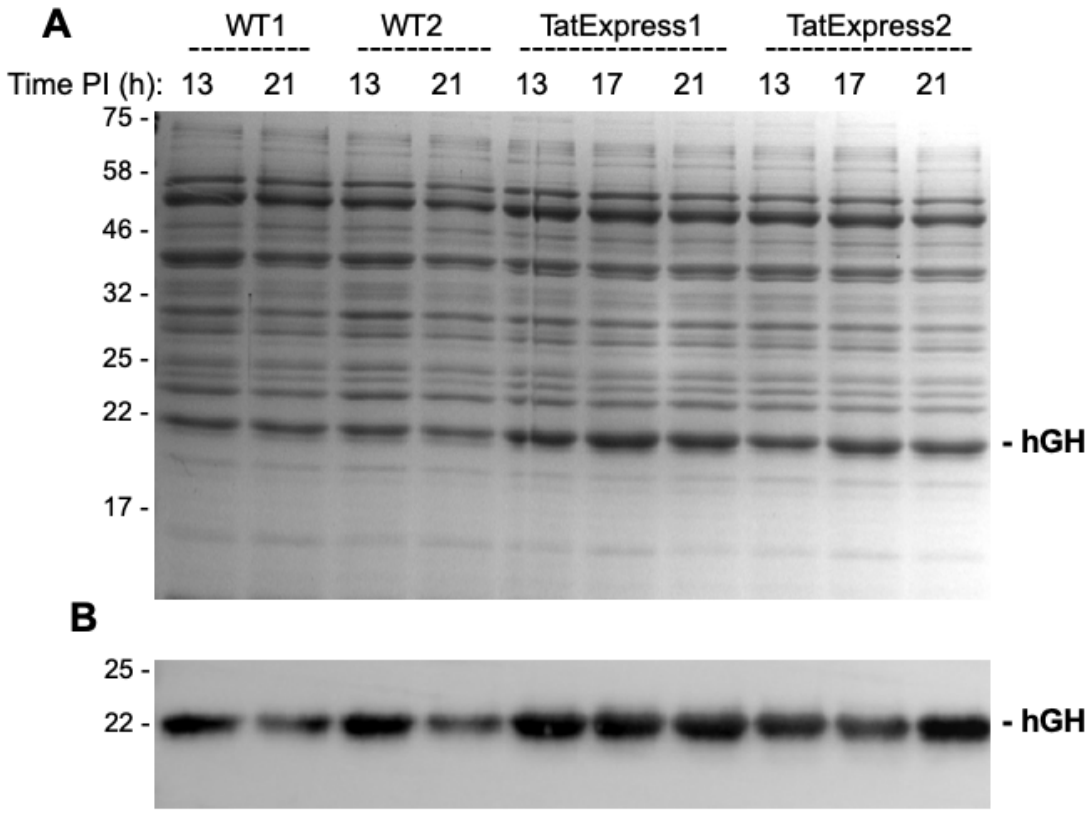

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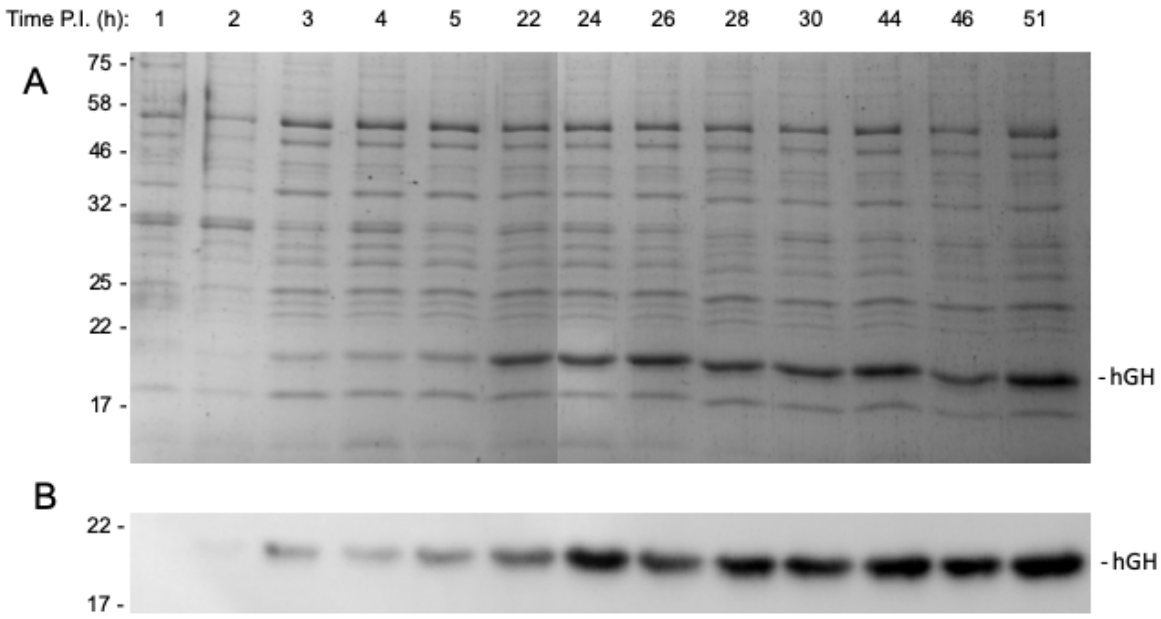

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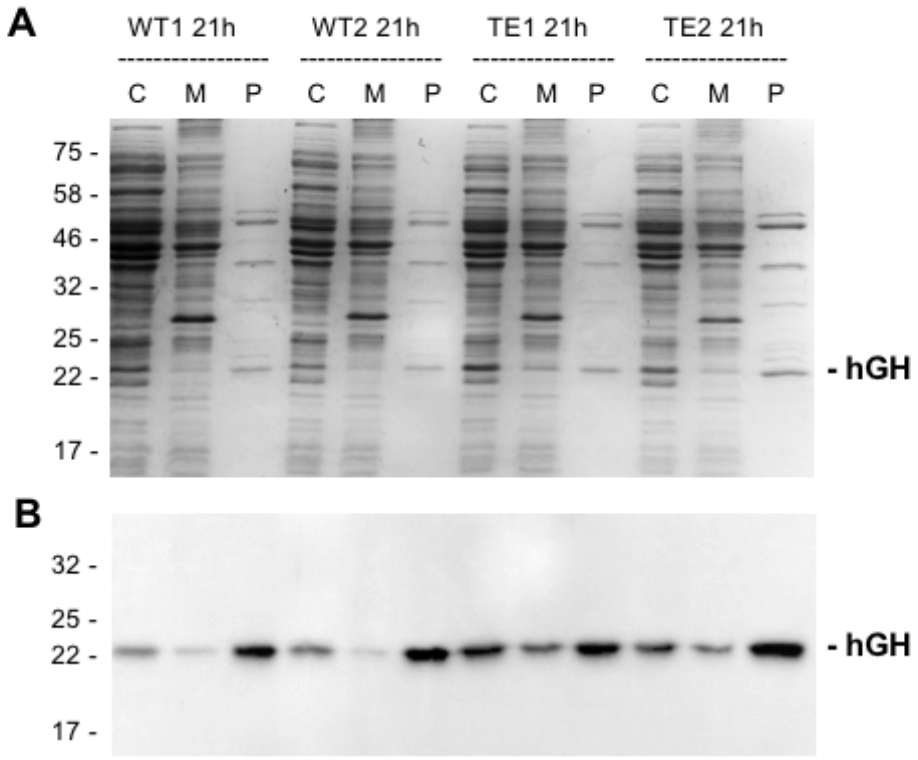

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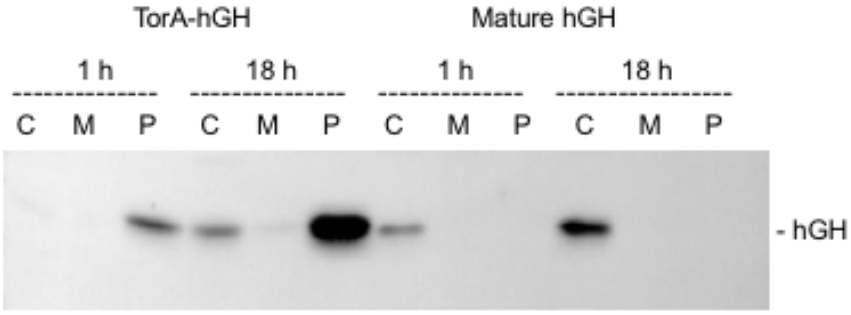

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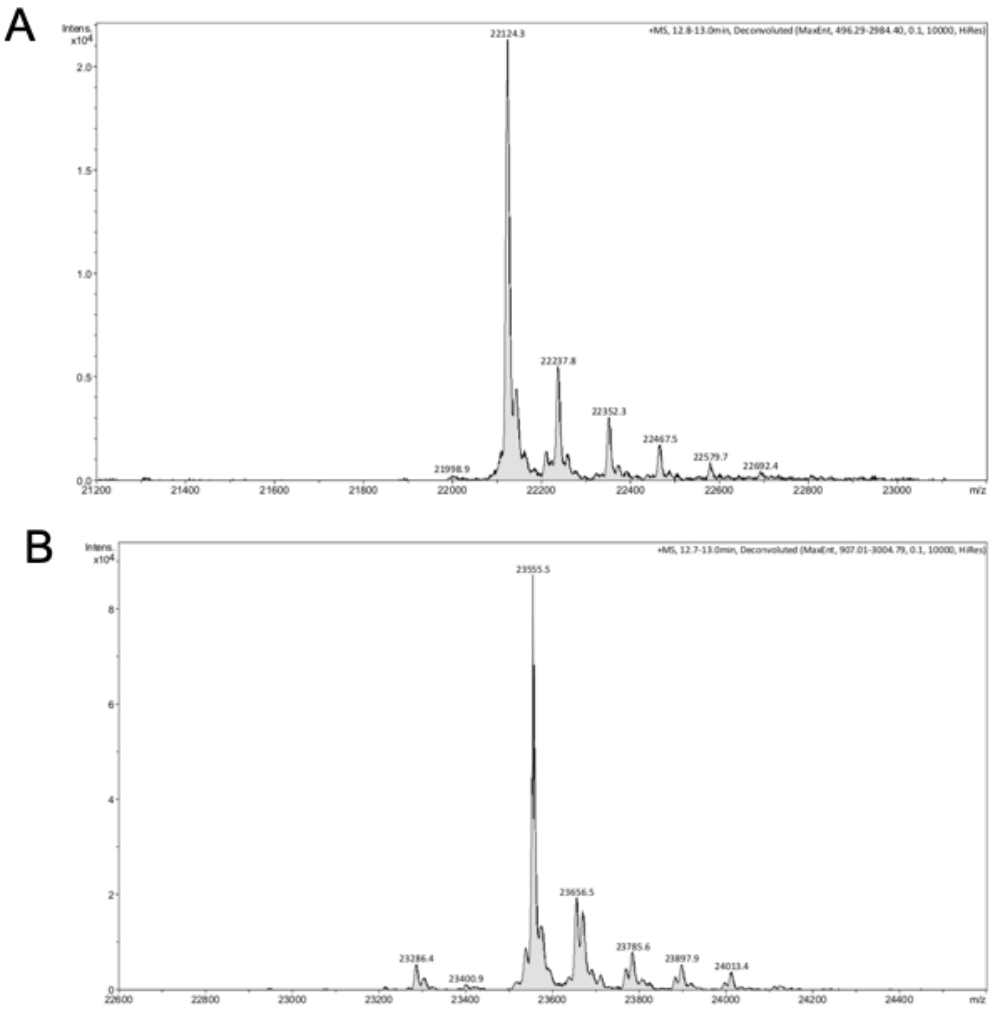

C

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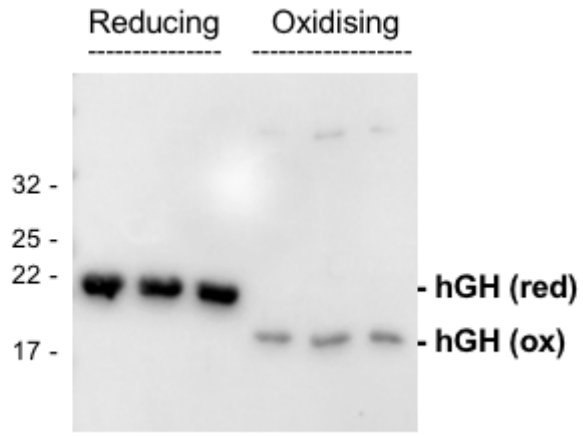

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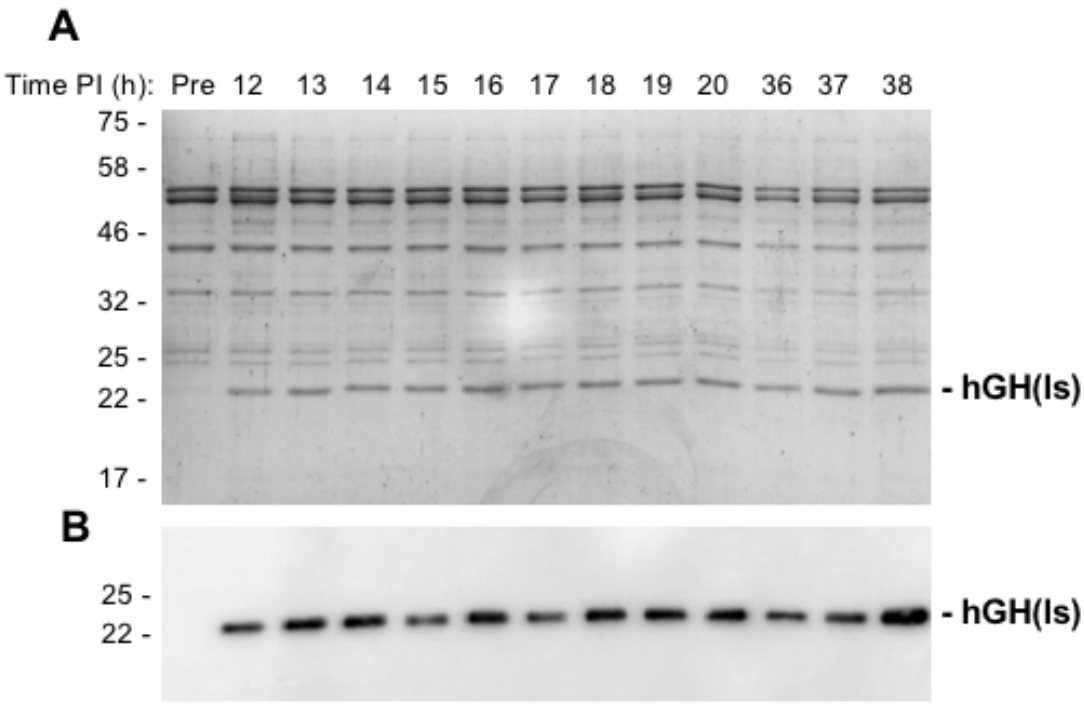

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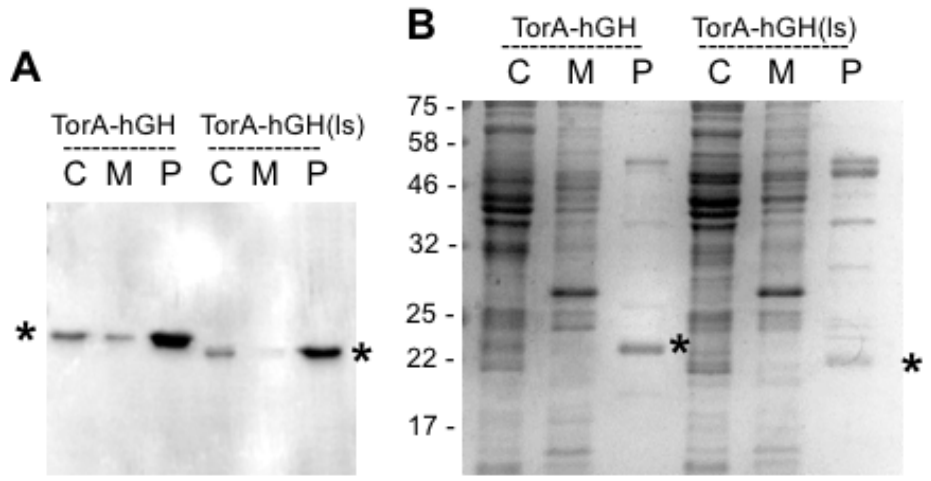

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