Engineering of the *Bacillus subtilis* PhoD signal peptide to direct high-level, Tat-specific export of a single-chain antibody fragment to the periplasm in *Eschericha coli*

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Research

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Abstract

Background : Numerous high-value proteins have been produced in E. coli, and a favoured strategy is to export the protein of interest to the periplasm by means of an N-terminal signal peptide. While the Sec pathway has been extensively used for this purpose, the Tat pathway has potential because it transports fully-folded heterologous proteins. Most studies on the Tat pathway have used the E. coli TorA signal peptide to direct export, because it is highly Tat-specific, unlike many Tat signal peptides which can also function as Sec signal peptides. However, the TorA signal peptide is prone to degradation in the cytoplasm, leading to reduced export rates in some cases. Here, we have tested a range of alternative signal peptides for their ability to direct Tat-dependent export of a single-chain antibody fragment (scFv).

Results : We show that the signal peptides of E. coli AmiC, MdoD and YcbK direct efficient export of the scFv by both the Tat and Sec pathways, which may be a disadvantage when Tat-specific export is required. The same applies to the Tat signal peptide of Bacillus subtilis PhoD, which likewise directs efficient export by Sec. We engineered the PhoD signal peptide by introduction of a Lys or Asn residue in the C-terminal domain of the signal peptide, and we show that this substitution renders the signal peptide Tat-specific. These signal peptides, designated PhoDk and PhoDn, direct efficient export of scFv in shake flask and fed-batch fermentation studies, reaching export levels that are well above those obtained with the TorA signal peptide. Culturing in ambr250 bioreactors was used to fine-tune the growth conditions, and the net result was export of the scFv by the Tat pathway at levels of approximately 1g protein/L culture.

Conclusions : The new PhoDn and PhoDk signal peptides have significant potential for the export of heterologous proteins by the Tat system.

Background

Numerous proteins are transported across the bacterial plasma membrane by one of two mainstream protein export pathways: the Sec and Tat pathways (reviewed in [1-5]). In *E. coli,* proteins destined for export by the Sec pathway are synthesised with N-terminal signal peptides that contain 3 domains: a positively-charged N-terminal (N) domain, hydrophobic core (H) domain and a more polar C-terminal (C) domain ending with an Ala-Xaa-Ala consensus domain that specifies cleavage by leader peptidase following export [4]. Substrates are transported across the membrane through a SecYEG channel in an unfolded state, primarily driven by the ATPase action of SecA [1].

Tat substrates are likewise synthesised with N-terminal targeting peptides, but translocation by the Tat pathway occurs by a completely different mechanism. Substrates are transported in a fully-folded form by a membrane-bound TatABC translocon that is driven by the proton motive force rather than ATP hydrolysis, and the available evidence (reviewed in [4,5]) indicates that the translocation mechanism is totally different, although the actual process remains poorly defined. Interestingly, there is mounting

evidence that the system is not only able to transport folded proteins, but is also inherently capable of preferentially selecting correctly-folded proteins for translocation (reviewed in [6]). A wide range of incorrectly folded proteins, including both natural Tat substrates and heterologous proteins, have been shown to be quantitatively rejected by the Tat system [e.g. 7-11].

The abilities of the Tat system have attracted increasing interest from a biotechnological standpoint. The system is able to export a wide range of heterologous proteins if a Tat-specific signal peptide is attached; examples include green fluorescent protein [12], alkaline phosphatase [7] and a series of biopharmaceuticals such as human growth hormone (hGH) and antibody single chain variable fragments [13]. Until recently it was unclear whether the Tat system could handle the large export rates required for industrial applications, but we recently showed that hGH could be exported at very high rates (several grams of protein per litre in fed-batch fermentation) in *E. coli* 'TatExpress' strains that over-express the *tatABC* operon to cope with high-level export [14].

The TorA signal peptide has been widely used for studies of the Tat pathway and for most of the published attempts to export heterologous protein substrates. This is partly due to the high export efficiency relative to many other Tat specific signal peptides, and partly because the signal peptide acts in a Tat-specific manner; in contrast, a number of other Tat signals can direct export of heterologous proteins *via* both the Tat and Sec pathways [15]. This reflects the broadly similar 3-domain structures of the two types of signal peptide. However, in our studies we have repeatedly observed that some constructs bearing the TorA signal peptide are subjected to degradation in the cytoplasm [e.g. 13]. In many cases, this leads to the production of what appears to be mature-size protein in the cytoplasm, and we have speculated that the TorA signal peptide may be highly susceptible to proteolysis, leading to the appearance of near-mature-size passenger protein in the cytoplasm. We therefore concluded that it should be possible to improve export rates through the use of a more effective and robust signal peptide. In this report we describe the design and testing of a new Tat signal peptide, based on the PhoD signal peptide from *Bacillus subtilis* that is able to direct the export of an antibody single chain variable fragment (scFv). The native PhoD signal peptide directs the export of an scFv by both the Sec and Tat pathways, but we show that an engineered form of this signal peptide is Tat-specific and able to direct the export of the scFv at significantly higher rates than those achieved with the TorA signal peptide.

Results

Several Tat signal peptides direct the export of a heterologous passenger protein by both the Tat and Sec pathways in E. coli

Previous studies on the export of heterologous proteins have shown that the TorA signal peptide is susceptible to proteolysis in the cytoplasm, which potentially leads to a loss of export efficiency when using the Tat system to export high-value proteins (e.g. [13]). We therefore tested the ability of other Tat signal peptides to direct high-efficiency export of biopharmaceuticals, and used a model biopharmaceutical that has shown to be exported by Tat in previous studies: a single chain variable fragment (scFv) [14]. We used three *E. coli* Tat signal peptides, taken from MdoD, AmiC and YcbK, and the Tat signal peptide from *Bacillus subtilis* PhoD, which is transported by the TatAdCd system in *B. subtilis* (reviewed in [16]).

In Fig. 1 the relative export efficiencies of several Tat signal peptides was investigated, using the scFv as a passenger protein. The constructs were expressed in shake flask cultures and synthesis was induced for several hours using IPTG. Afterwards, the cells were fractionated to yield cytoplasm, membrane and periplasm samples, which were analysed by immunoblotting using antibodies to the C-terminal His tag on the scFv. To test whether export is Tat-specific, export assays were carried out both in wild type cells and a *tat* null mutant (*Δtat*). In the control test, the TorA signal peptide exports scFv in wild type cells, with the mature scFv form evident in the periplasm (P). A protein migrating with about the same mobility as mature scFV is detected in the cytoplasm (C), illustrating the point that cleavage of the TorA signal peptide readily occurs in this compartment; the full precursor form is barely detectable. A mixture of precursor and the near-mature-size scFv is found in the membrane fraction (M). Essentially no export is observed in *Δtat* cells, confirming that export is Tat-specific.

Similar tests were carried out using AmiC-scFv, MdoD-scFv, YcbK-scFv and PhoD-scFv, and the data show that these constructs are all efficiently exported in both wild type and *Δtat* cells. This strongly indicates that export is taking place by both the Tat and Sec pathways. Export is more efficient in wild type cells, but given that these export levels are due to a combination of Tat- and Sec-dependent export, it is clear that Tat-dependent export is by no means particularly dominant over Sec-dependent export. Several of these signal peptides have been shown to mediate Sec-dependent export in previous studies (e.g. [15]) but these data provide a clear picture that the level of Sec-dependent export can be substantial.

Engineering of modified PhoD signal peptides to confer Tat-specificity

We considered it important to generate an efficient Tat-specific signal as an alternative to the TorA signal, and modified the PhoD signal peptide because it provided high levels of export in our initial tests using scFv as a passenger protein (see Fig. 1). The sequence of the native PhoD signal peptide is shown in Fig. 2, and in an effort to render the signal peptide Tat-specific, we replaced the Ala residue at the -6 position

(arrowed) with Ile, Lys and Asn, generating signal peptides designated PhoDi, PhoDk and PhoDn, respectively (see sequences in Fig. 2). Leader peptidase, the enzyme responsible for the removal of signal peptides after translocation, cleaves after a consensus sequence in which the -1 and -3 residues are invariably short-chain [17], and the native cleavage site in *B. subtilis* ends with VNA as shown in Fig. 2 (shown by a second arrow).

We reasoned that the introduction of a Lys at the -6 position in the PhoDk signal peptide should reduce or block export by the Sec pathway because a number of studies have shown that the presence of basic residues in the C-terminal domain of a signal peptide prevents transport by Sec [18,19]. The introduction of Ile and Asn (PhoDi and PhoDn) was also carried out, although this was judged less likely to confer complete Tat-specificity. Fig. 2A shows initial export assays using the modified PhoD signal peptides in WT cells. After induction of synthesis, cells were fractionated into spheroplast and periplasm samples (S, P) and the results show that all three modified signal peptides direct efficient export of the scFv into the periplasm (P).

The PhoDn and PhoDk signal peptides direct Tat-specific export of scFv

We next tested whether the modified PhoD signal peptides are Tat-specific, and the data are shown in Fig. 2B. The three scFv constructs were expressed in *Δtat* cells in which all Tat components are missing, and after induction of synthesis for 3 h the cells were fractionated to generate cytoplasm, membrane and periplasm samples (C, M, P) which were analysed by immunoblotting to detect scFv. The results are clear-cut: PhoDi-scFv is exported to the periplasm at high levels and processed to the mature size, indicating efficient translocation by Sec, whereas PhoDn-scFv and PhoDk-scFv are not exported to any detectable extent, showing that these signal peptides are Tat-specific. In fact, PhoDn-scFv and PhoDk-scFv are not detected in the cytoplasm or membrane fractions of the *Δtat* cells either, even in this immunoblot which is highly exposed to detect even low signal levels, which is surprising given that the precursor form was clearly detectable in the cytoplasm of the wild type cells as shown in Fig. 2A. This point was further tested in Fig. 2C, which shows similar export assays carried out in *Δtat* cells after a range of induction times (60, 90 and 120 min post-induction). The same result is obtained: PhoDi-scFv is efficiently exported (presumably by Sec) whereas the other two constructs are not detected at any time and thus appear to be rapidly degraded in the cytoplasm. We conclude that the PhoDn-scFv and PhoDk-scFv constructs are particularly unstable in the cytoplasm of *Δtat* cells, for reasons that are completely unclear.

PhoDn-scFv is exported with high efficiency during fed-batch fermentation

The primary aim of this study was to generate a signal peptide that is capable of directing high-level, Tat-specific export in industrial applications, and further expression and export tests were carried out in fed-batch fermentation systems that are much more representative of those used in industrial processes. We compared the export of scFv bearing the TorA and PhoDn signal peptides, and first used INFORS fed-batch fermentation systems as detailed in Methods. The synthesis of TorA-scFv and PhoDn-scFv was induced using IPTG and samples were analysed at time points from 12 to 39 hours post induction. The cells were fractionated and periplasmic samples were analysed by immunoblotting to detect the scFv, and on Coomassie-stained gels to analyse the periplasmic proteomes. The immunoblots in Fig. 3 show that TorA-scFv is steadily exported to the periplasm during the initial part of the culture but the levels decline at later time points (36-39 h). The stained gels show that the scFv is exported at high levels during the first phase of the culture, becoming one of the most abundant periplasmic proteins within a few hours of induction. Thereafter, the levels decline and there is evidence that at least one degradation product is present under the band representing periplasmic scFv.

PhoDn-scFv is also exported with high efficiency and it is notable that the levels of scFv per cell rise throughout the culture run, with no signs of degradation at later time points. Analysis of the stained gel shows that the scFv is a prominent periplasmic protein by the end of the induction period, and there is no indication of breakdown products. The levels of periplasmic scFv exceed those observed during export of TorA-scFv, and these data thus show that the PhoDn signal peptide is more effective than the TorA signal peptide for the export of this scFv under these conditions.

To further compare the relative merits of the PhoDn and TorA signal peptides, we carried out further studies using the ambr250 automated bioreactor. The ambr250 modular is a benchtop bioreactor system for parallel cell culture in single-use vessels with 100-250 mL working volumes. It is used extensively by industry and the system comprises a series of benchtop modules enabling up to 8 bioreactors to be operated in parallel and a control module with system software capable of automating feed additions. We used the system to test whether the PhoDn signal peptide gives a consistent improvement over the TorA signal peptide for the export of the scFv, and first compared the export of TorA-scFv and PhoDn-scFv using a single concentration of IPTG (0.36 mM) over a 19 h time course. Fig. 4 shows the periplasmic scFv analysed using immunoblots and Commassie-stained gels; the data clearly show that levels of exported scFv are far higher with the PhoDn signal peptide than with the TorA peptide. The data again show that in the PhoDn-scFv export samples, the scFv is an abundant periplasmic protein by the end of the time course study.

We next used the ambr250 system to systematically determine the optimal IPTG concentration for export, using 4 different concentrations of IPTG to provide a range of expression levels. In this experiment, samples were analysed over a more extended period: 12-38 h after induction. Fig. 5 shows immunoblots of the scFv levels in the periplasm, and it is clear that the TorA-scFv is present at relatively similar levels per cell throughout the time course, with evidence that the levels decline towards the end of the induction period in some cases (particularly the samples from the cultures induced with IPTG concentrations of 0.1, 0.4 and 0.8 mM). In contrast, the PhoDn-scFv is present at per-cell levels that steadily increase in some cases (IPTG concentrations of 0.4 and 0.8 mM) or remain fairly constant in others (0.1 and 1.0 mM IPTG).

To effectively compare the export efficiencies, we analysed the 38 h periplasm samples from each of the ambr250 runs on the same blot (Fig. 6A). Here, the data clearly show that the PhoDn signal peptide directs much higher export efficiency than the TorA signal peptide. Peak export of PhoDn is observed with 1.0 mM IPTG, although all IPTG concentrations support efficient export. The periplasmic fractions from the 1.0 mM IPTG runs were analysed on Coomassie-stained gels (Fig. 6B) which shows the periplasmic scFv to be a prominent periplasmic protein by the end of the induction period.

We have purified the scFv from periplasmic fractions in order to quantify the levels of scFv protein that can be generated from this type of fermentation process, but this particular scFv does not bind well to Protein L (commonly used for purification of scFvs) or to affinity resins that are used to bind His-tagged proteins. We believe that the C-terminal His tag is buried within the native structure and inaccessible. Most of the protein is thus lost during purification, which makes quantification difficult. However, the scFv has the same His tag as that present on hGH which which was exported at levels of 2-5 g/L culture in a previous study [14], and we have therefore used semi-quantitiative immunoblotting with the scFv and hGH run on the same gels and blots. This indicates that the levels of scFv exported with the PhoDn signal peptide reach approximately 1g protein per litre of culture, while the TorA-scFv is exported at significantly lower levels.

Discussion

Recent studies have shown that the Tat pathway can export human growth hormone at levels of several g/L in fed-batch fermentation cultures of *E. coli* 'TatExpress' strains that simultaneously over-express the Tat machinery [14]. Clearly, this platform is capable of high-level protein secretion to the periplasm, but previous work has already pointed to potential limitations in the current platform. In particular, the TorA signal peptide is very widely used because it delivers high-level, Tat-specific secretion, yet appears to have the disadvantage that it is prone to proteolytic degradation in the cytoplasm. This leads to a situation in which export efficiency is heavily influenced by the relative rates of export *vs* degradation. We commonly observe the presence of 'pseudo-mature' size protein in the cytoplasm when expressing contructs bearing

a TorA signal peptide, which suggests that the precursor proteins are being cleaved near the signal peptide-mature protein junction [13].

We therefore set out to identify an alternative signal peptide, and additionally sought to test whether another industrially relevant protein (in this case an scFv) could be exported at high levels by Tat. Studies on the other signal peptides used in this study provide an interesting comparison. While the TorA signal peptide is extremely Tat-specific, the *E. coli* AmiC and MdoD signal peptides direct efficient Sec-dependent transport in *Δtat* cells, as does the PhoD signal peptide from *B. subtilis.* In these cases, export efficiency is approximately double in wild type cells and our conclusion from these results is that the signal peptides are effective as both Sec or Tat signal peptides. A previous study has shown that a wide range of Tat signal peptides can support Sec-dependent export using using an assay in which growth of *malE* -defective cells was dependent on export of signal peptide-maltose binding protein fusion proteins [15]. Here, we have clearly shown that the above signal peptides can direct high rates of Tat-independent (presumably Sec-dependent) transport of this scFv.

The wild type *B. subtilis* PhoD signal peptide directs very efficient export of the scFv but is clearly directing transport by both the Tat and Sec pathways. However, a single amino acid substitution renders the signal peptide Tat-specific, and the engineered PhoDn and PhoDk signal peptides thus represent attractive alternatives to the well-known TorA signal peptide. In this study we have set out to systematically test the capabilities of the PhoDn signal peptide, using shake-flask, 'standard' fed-batch fermentation and the automated ambr250 fed-batch systems. In each case, the export of the PhoDn-scFv was compared directly with that of a TorA-scFv fusion protein and the data show that the PhoDn signal peptide delivers a significantly enhanced level export of the scFv. By the end of the fermentation runs, the scFv is a highly abundant periplasmic protein, demonstrating that the new engineered signal peptide can be used for high-level export of this heterologous protein by the Tat pathway.

It should be noted that we have not tested the 'quality'of the exported proteins, in terms of folding fidelity or activity. However, several heterologous proteins (including an scFv)have been exported by Tat and shown to be active in previous studies [7,11,12,13] and there is no obvious reason why export mediated by the PhoDn signal should be different. Moreover, a range of studies have shown that the Tat system is predisposed to export correctly folded proteins [5-18, 10].

Conclusions

The study sheds further light on the abilities of the Tat system to direct export of high-value proteins to the *E. coli* periplasm. The availability of a new, engineered, Tat-specific signal peptide means that yields

of periplasmic scFv are much higher and this tool is a potentially valuable complement to the engineered 'TatExpress' cells that are capable of higher rates of protein transport due to their elevated levels of TatABC.

Methods

Strains and Plasmids

DNA fragments containing His-tagged TorA-scFv and His-tagged PhoD-scFv were cloned into a pEXT22 expression vector [20] using overlap extension PCR cloning [21] (Supplementary table S1) to create constructs pKRK5 and pKRK12 respectively. PhoDn, PhoDk and PhoDi signal peptide mutants were generated by site directed mutagenesis using 5' phosphorylated primers (Supplementary table S1) and pKRK12 vector, containing wild type PhoD signal peptide, as a template, to mutate the Ala residue at position 51 to Asn, Lys or Ile. Plasmids were transformed into *E. coli* MC4100, or W3110 TatExpress cells [22] for protein production. Strains and plasmids are listed in Table 1.

Strain/Plasmid	Description	Source
MC4100	E. coli K-12 strain. ra^R, F2 araD139 DlacU169 rpsL150 relA1 flB5301 deoC1 ptsF25 rbs^R	[23]
ΔtatABCDE	MC4100 strain lacking *tatABCDE* genes, Ara^R	[23]
W3110 TatExpress	E. coli K-12 strain. F- λ- rph-1 INV(rrnD, rrnE); ptac promoter upstream of *tatABCD*	[14]
pEXT22	Protein over-expression vector carrying kanamycin resistance	[20]
pKRK5	pEXT22 TorA-scFv-His6	This study
pKRK12	pEXT22 PhoD-scFv-His6	This study
pKRK57	pEXT22 PhoDi-scFv-His6	This study
pKRK58	pEXT22 PhoDk-scFv-His6	This study
pKRK59	pEXT22 PhoDn-scFv-His6	This study

Table 1. Strains and plasmids used in this study.

Primer name	Sequence (5’ – 3’)
Primer 7A F	ATGAACAATAACGATCTCTTTC
Primer 7C F	GAGCTCGGTACCCTACCACAGAGGAACATGT
Primer 9B R	AAATCTAGTCGGATCTCAGT
Primer 9D R	CAGAAGCTTGCATGCCTGCAGGTCGACTCTAGA
Primer 10A F	ATGGCCTATGATAGCCG
Primer 10B R	ATGAAAGTTCGGTGCTG
Primer 10D R	CAGACTCCACCAGCTGCACCTCGGCCAT
PhoD Mut R	P-TGCAATGGTCAGACCCAGGCTC
PhoD Mut Ile F	P-CAGAGCGTTGGTATTTTTGAAGTTAATGC
PhoD Mut Lys F	P-CAGAGCGTTGGTAAATTTGAAGTTAATGC
PhoD Mut Asn F	P-CAGAGCGTTGGTAACTTTGAAGTTAATGC

Supplementary table 1. DNA primers used in this study. P- denotes 5’ phosphorylation.

Shake flask cell culture

Shake flask expression of recombinant proteins was performed by first creating a pre-inoculate culture by taking a single colony from the transformed plate and inoculating into 5 mL LB medium with ampicillin and growing for 16 hours at 30 °C, 200 rpm in a shaking incubator. The following day, 50 mL LB medium containing 100 µg/mL ampicillin was inoculated with the pre-inoculate culture to an optical density (OD_{600}) of 0.05, then grown at 30 °C, 200 rpm for approximately 3 hours until the OD_{600} of the culture reached 0.4-0.6. Once reached, expression was induced by adding 100 µM IPTG to the culture, then the cultures continued to grow at 30 °C, 200 rpm, harvesting cell samples at 3 hours and 21 hours after induction. At the specified timepoints, the OD_{600} of the culture was determined and a volume of culture equivalent to 10 OD_{600} was harvested by centrifugation at 3000 rpm for 10 minutes at 4 °C. The cell pellet was resuspended in 1 mL resuspension buffer (50mM Tris-acetate, 2.5 mM EDTA, pH 7.0) then lysed by sonication in ice at all times, using amplitude 8.0 (Soniprep 150plus, Sanyo Gallenkamp, Loughborough, UK) for 4-6 rounds consisting of 10 seconds sonicating, alternated with 10 seconds resting. Lysed suspensions were centrifuged at 14,000 rpm for 15 minutes at 4 °C and the supernatant kept as “Soluble” cell fraction, while the pellet was resuspended in a further 1 mL resuspension buffer and kept as “Insoluble” cell fraction, for analysis by SDS PAGE.

Fermentation - Infors bioreactors

Transformants were picked from selective plates and grown in 5ml of 6xPY media with 50 µg/mL of kanamycin at 30°C with shaking at 250 rpm for 6 hours. 2 mL of the 6xPY overday cultures were used to inoculate 200 mL overnight cultures in SM6Gc media with 50 µg/mL of kanamycin. Overnight cultures were grown at 30°C with shaking at 250 rpm. A volume equivalent to 150 OD_{600} of overnight culture was used to inoculate 500 mL of SM6Gc in the 1.5L Infors Minifors 2 bioreactors (Infors HT). Dissolved oxygen was maintained at 40% saturation by sparging of compressed air, and pH maintained at 7.0 with the addition of 25% (v/v) sulphuric acid and 25% (v/v) ammonia under PID control. Cultures were grown at 30°C until airflow and stirring speed were maximal and then reduced to 25°C for the remainder of the run. Cell density was measured every two hours by optical density at 600nm. Cultures were supplemented with $MgSO_4$ at OD_{600} 38-42 (8 mL/L of 1M $MgSO_4$), and with Na_2HPO_4 at OD_{600} 54-58 (5 mL/L of 232.8 g/L Na_2HPO_4) and at OD_{600} 66-70 (7 mL/L of 232.8 g/L Na_2HPO_4). A feed of 80% (w/w) glycerol was started concurrently with the second Na_2HPO_4 addition at a constant feed rate of 0.01 mL/min. The cultures were induced with IPTG at concentrations specified in the Figures when the cultures reached OD_{600} 75.

Fermentation – Ambr250 bioreactors

Transformants were picked from selective plates and grown in 5 ml of 6xPY media with 50 µg/mL of kanamycin at 30°C with shaking at 250 rpm for 6 hours. 2 mL of the 6xPY overday cultures were used to inoculate 500mL overnight cultures in SM6Gc media with 50µg/mL of kanamycin. Overnight cultures were grown at 30°C with shaking at 250 rpm. A volume equivalent to 60 OD_{600} of overnight culture was used to inoculate 200 mL of SM6Gc in the 250 mL ambr250 bioreactors (Sartorius Stedim). Each overnight culture was used to inoculate four bioreactors and the bioreactors were loaded into the control units in pairs consisting of one TorA-scFv and one PhoDn-scFv culture. Dissolved oxygen was maintained at 40% saturation by sparging of compressed air, and pH maintained at 7 with the addition of 25% (v/v) sulphuric acid and 25% (v/v) ammonia under PID control. Cultures were grown at 30°C until airflow and stirring speed were maximal and then reduced to 25°C for the remainder of the run. Cell density was

measured every two hours by optical density at 600 nm. Cultures were supplemented with $MgSO_4$ at OD_{600} 38-42 (8 mL/L of 1M $MgSO_4$), and with Na_2HPO_4 at OD_{600} 54-58 (5 mL/L of 232.8 g/L Na_2HPO_4) and at OD_{600} 66-70 (7 mL/L of 232.8 g/L Na_2HPO_4). A feed of 80% (w/w) glycerol was started concurrently with the second Na_2HPO_4 addition at a constant feed rate of 0.01 mL/min. Cultures were induced in their respective pairs with IPTG concentrations as detailed in the Figures.

Periplasmic Fractionation and Protein Purification

Cells equivalent to 10 OD_{600} were taken and fractionated into periplasmic (P) and spheroplast fractions by cold osmotic shock with lysozyme and EDTA. Cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C and resuspended in 500 µL of ice-cold Buffer 1 (500 mM sucrose, 100 mM Tris-acetate pH 8.2, 5 mM EDTA pH 8.0). 500 µL of dH_2O was added, followed by 40 µL of hen egg white lysozyme (1 mg/mL). Samples were incubated on ice for 5 min and 20 µL of $MgSO_4$ (1M) was added to stabilize the inner membrane. Samples were centrifuged at 14,000 rpm for 2 minutes at 4°C and 750 µL of supernatant was taken as the periplasmic fraction and stored at -20°C. The remaining supernatant was discarded and the pellet washed by resuspension in 1 mL of Buffer 2 (50 mM Tris-acetate pH 8.2, 250 mM sucrose, 10 mM $MgSO_4$) and spun at 14,000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was stored at -20°C.

Proteins were purified by nickel IMAC affinity to the C-terminal hexahistidine tag. 20 mL of fermentation culture was taken and centrifuged at 4000 rpm for 20 min at 4°C. The weight of the cell pellet was recorded and the pellet was resuspended in 10 mL/g of ice-cold Buffer 1 without EDTA. Samples were incubated on ice for 20 min before the addition of 10 mL/g of dH_2O and incubation on ice for a further 30 minutes. Samples were centrifuged for 20 min at 14,000rpm and 4°C (Beckman Avanti J- 25, JA 25.5 rotor). Supernatant was taken as crude periplasmic extract and purified using an ÄKTA™ pure protein purification system and a HisTrap HP histidine-tagged protein column (GE Healthcare, Buckinghamshire, UK). The 20% ethanol storage solution was washed off with 5 column volumes (CV) of milliQ H_2O and

equilibrated with 5CV of equilibration buffer (50 mM sodium phosphate pH 7.2-7.6, 150 mM sodium chloride, 10 mM imidazole). Periplasmic extract was loaded and the flow through (FT) was collected. The column was washed with 10CV of equilibration buffer and a sample collected as Wash (W). The bound scFv was eluted by linear gradient elution from 10-250 mM Imidazole over 5CV with an additional 2CV at 250 mM Imidazole. Elution fractions were run on SDS-PAGE and stained with Coomassie blue to identify fractions containing purified scFv.

Analysis by Coomassie stained gels and Western blot

Samples were resolved by reducing SDS-PAGE (15% acrylamide) and analysed using Coomassie blue staining and Western blotting. Protein was transferred to a PVDF membrane by wet Western blotting with electrophoresis for 1h at 80V, 300A. The membrane was incubated at 4°C overnight in blocking solution (5% (w/v) skimmed milk powder in 50mL 1xPBS and 0.1% Tween20 (PBS-T)). The membrane was washed 3 times for 5 minutes with PBS-T and incubated in primary antibody (3.5 µL anti-His (C-term) (Life Technologies, CA, USA) in 20 mL PBS-T with 5% (w/v) bovine serum albumin (Sigma Aldrich)) for 1 hour at room temperature. Membranes were washed 3 times for 5 minutes with PBS-T before incubation with secondary antibody (4 µL anti-Mouse HRP conjugate in 20 mL PBS-T) for 1 hour at room temperature. Membranes were washed 5 times for 5 minutes with PBS-T and immunoreactive bands were detected using an enhanced chemiluminescence kit (BioRad) following manufacturers instructions. Blots and gels were visualised and imaged using a BioRad Gel Doc imager and ImageLab v4.1 Software. Quantitation of scFv yield was performed using anti-His ELISA (Genscript) according to manufacturer's instructions and comparative densitometry of band intensities was performed using ImageLab software, with a known quantity (13.5 mg/mL) of purified hGH as a reference.

Declarations

- Ethics approval and consent to participate. Not applicable.
- Consent for publication. Not applicable.
- Availability of data and materials. All data generated or analysed during this study are included in this published article.

- Competing interests. The authors declare that Sartorius Ltd provided expertise in the operation of the ambr250 fermentation system used in this study. This assistance was recognized by the inclusion of Dr Lara Nascimento, a Sartorius employee, in the author list.
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- Author contributions. CJ, KLR, ARP and KLW made substantial contributions to the acquisition, analysis, and interpretation of data. LN made substantial contributions to the design of the work, in particular the ambr250 sytudies; CR designed the study, made contributions to the interpretation of the work and drafted the manuscript.

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Figures

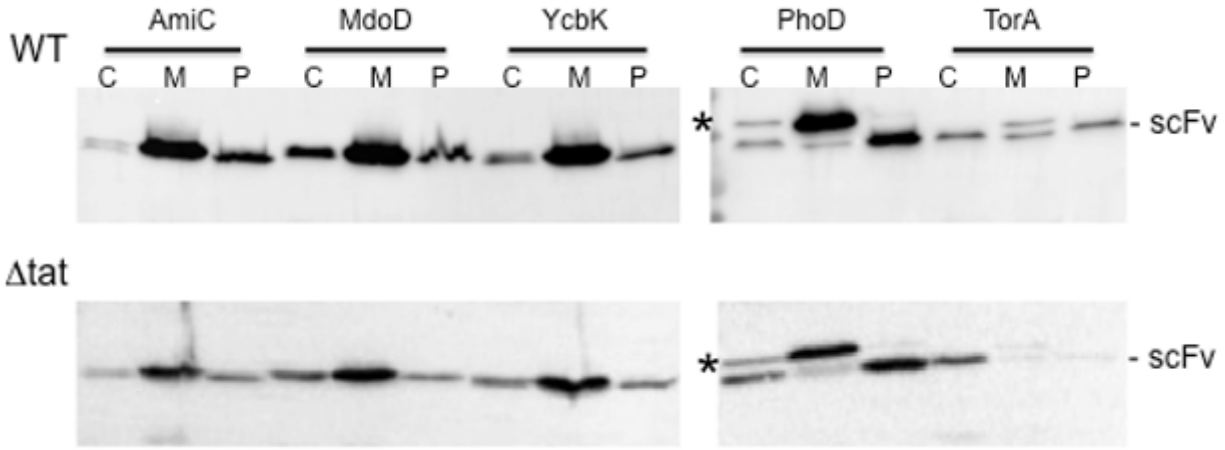

Figure 1

Tat signal peptides direct the export of an scFv with differing efficiencies and with differing degrees of pathway specificity. Constructs comprising the signal peptides of TorA, MdoD, AmiC (all derived from E. coli) and B. subtilis PhoD linked to an scFv were expressed in MC4100 wild type or ΔtatABCDE strains (denoted WT, Δtat). After induction for 3 h with IPTG, cells were fractionated and samples of cytoplasm, membrane and periplasm fractions (C, M, P) were analysed by SDS-PAGE. The scFv was detected using antibodies to the His tag on the C-terminus. Mobility of mature forms of scFv are indicated ('scFv') and an asterisk denotes the full precursor form.

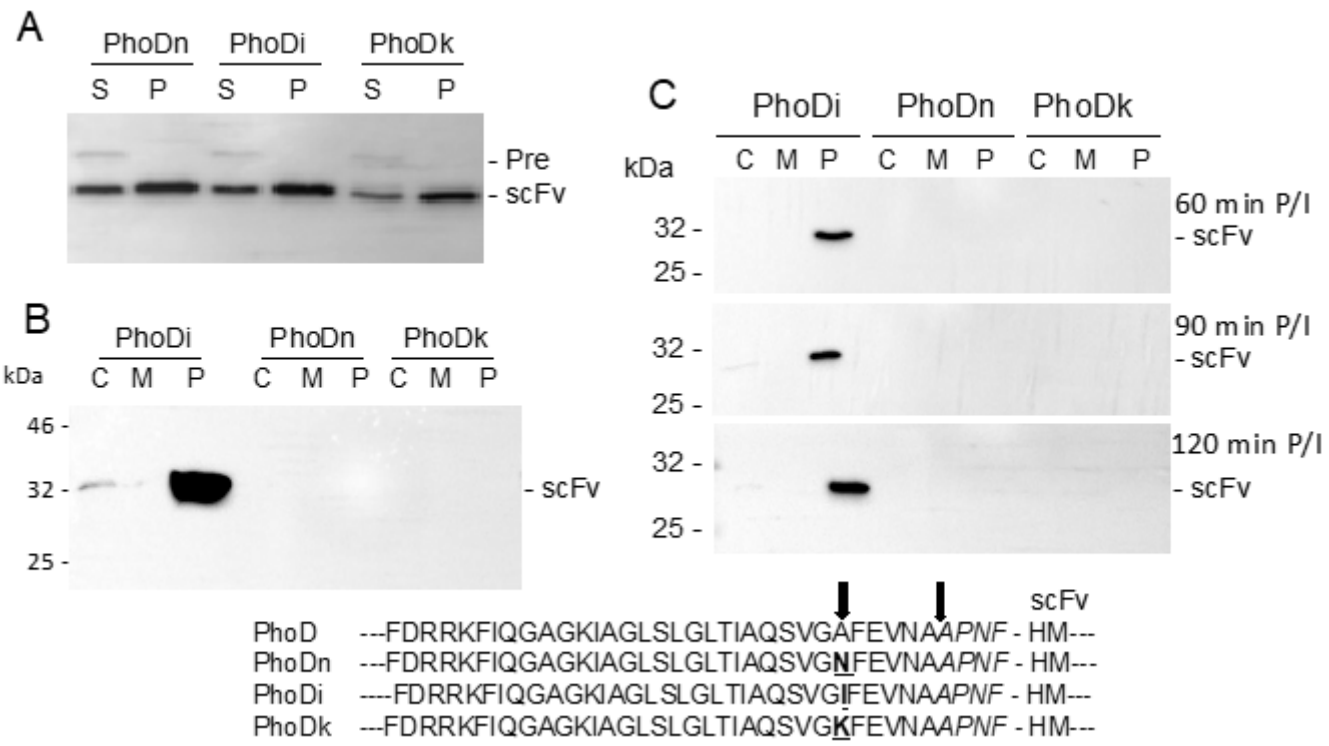

Figure 2

The scFv is exported in a Tat specific manner by 2 engineered PhoD signal peptides. The PhoD-scfv construct was engineered by substitution of the -6 Ala residue in the signal peptide (left hand arrow above the peptide sequences) with Lys, Asn and Ile, generating PhoDk, PhodN and PhoDi peptides, respectively. A: the constructs were expressed in WT MC4100 cells and after 3h induction with IPTG cells were fractionated into spheroplast and periplasm fractions (S, P) which were immunoblotted to detect the scFv. B: the constructs were expressed under the same conditions in Δtat cells and cells were fractionated into cytoplasm, membrane and periplasm fractions (C, M, P). C: the constructs were expressed in Δtat cells and samples were fractionated and analysed 60, 90 and 120 min post-induction (P/I). All gels were immunoblotted using antibodies against the His tag on scFv. Mobilities of molecular mass markers (in kDa) are indicated. Mobilities of molecular mass markers (in kDa) are shown on the left.

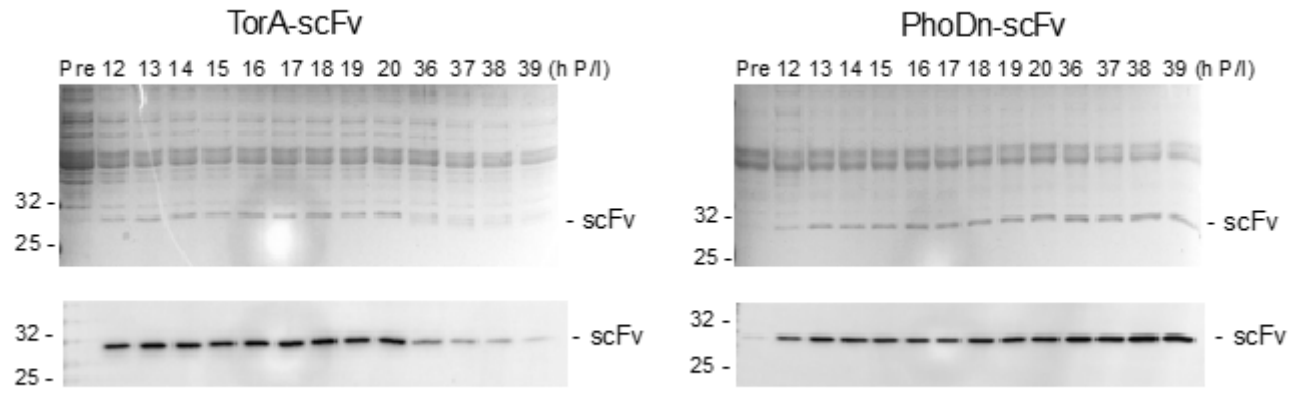

Figure 3

PhoDn-scFv is exported at high levels during fed-batch fermentation. PhoDn-scFv and TorA-scFv were expressed in E. coli W3110 'TatExpress' cells in INFORS bioreactors under fed-batch fermentation conditions. Samples were analysed pre-induction ('pre') and after times (in h) post induction with IPTG (P/I). Cells were fractionated to generate periplasm samples, which were analysed using Coomassie-stained gels (upper panels) or immunoblotting to detect the scFv (lower panels).

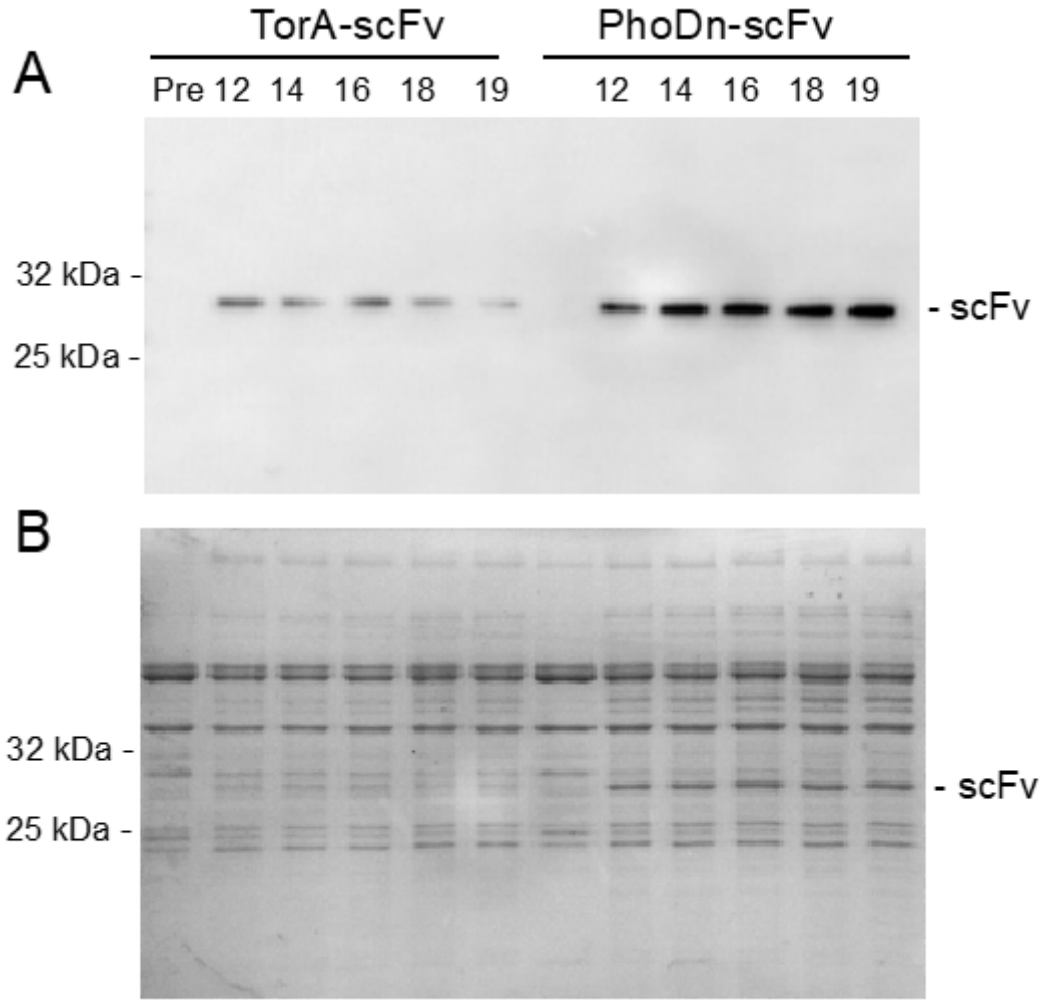

Figure 4

Fed-batch fermentation analysis of TorA-scFv and PhoDn-scFv export using an automated ambr250 bioreactor. TorA-scFv and PhoDn-scFv were expressed in E. coli TatExpress cells in parallel using an ambr250 automated bioreactor, and synthesis was induced using 0.36 mM IPTG. Samples were analysed pre-induction (Pre) and after induction times shown (in h). Periplasmic samples were analysed using Coomassie stained gels and immunoblotting to detect the scFv.

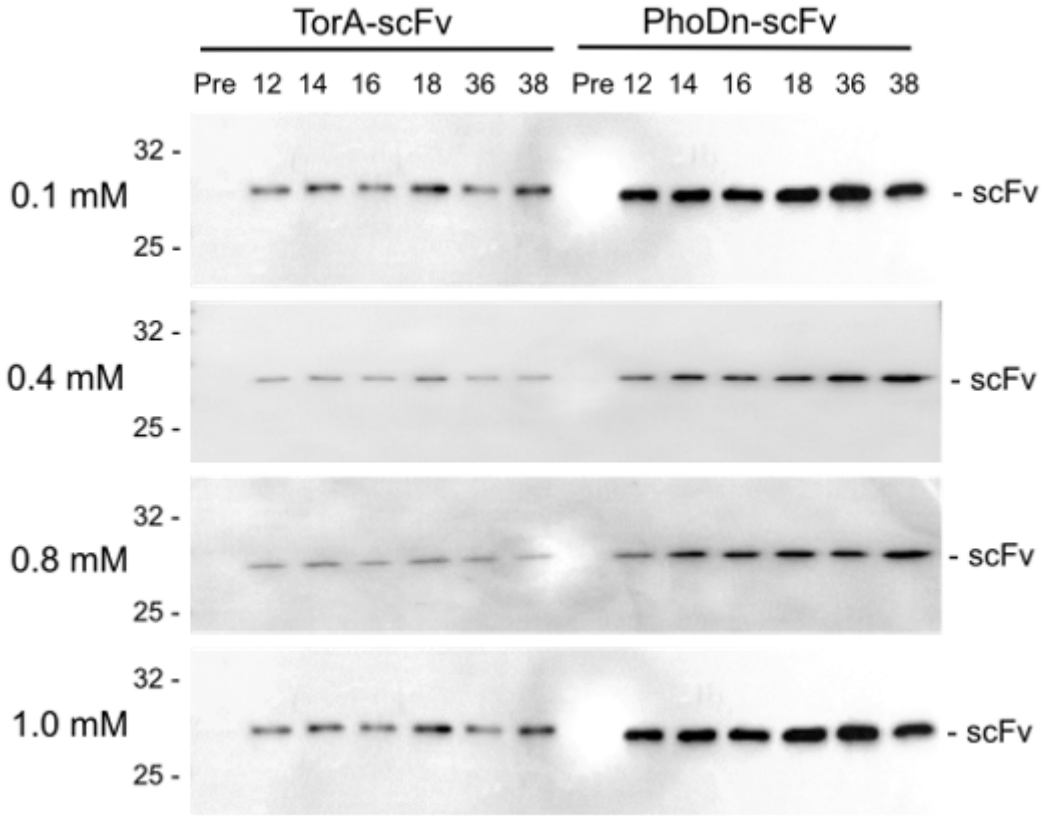

Figure 5

Optimisation of scFv export using an ambr250 bioreactor. TorA-scFv and PhoDn-scFv were expressed in TatExpress cells using an ambr250 bioreactor, and parallel cultures were induced using 0.1, 0.4, 0.8 and 1.0 mM IPTG. Periplasmic samples were analysed by immunoblotting to detect the scFv before induction (Pre) and after induction times shown (in h).

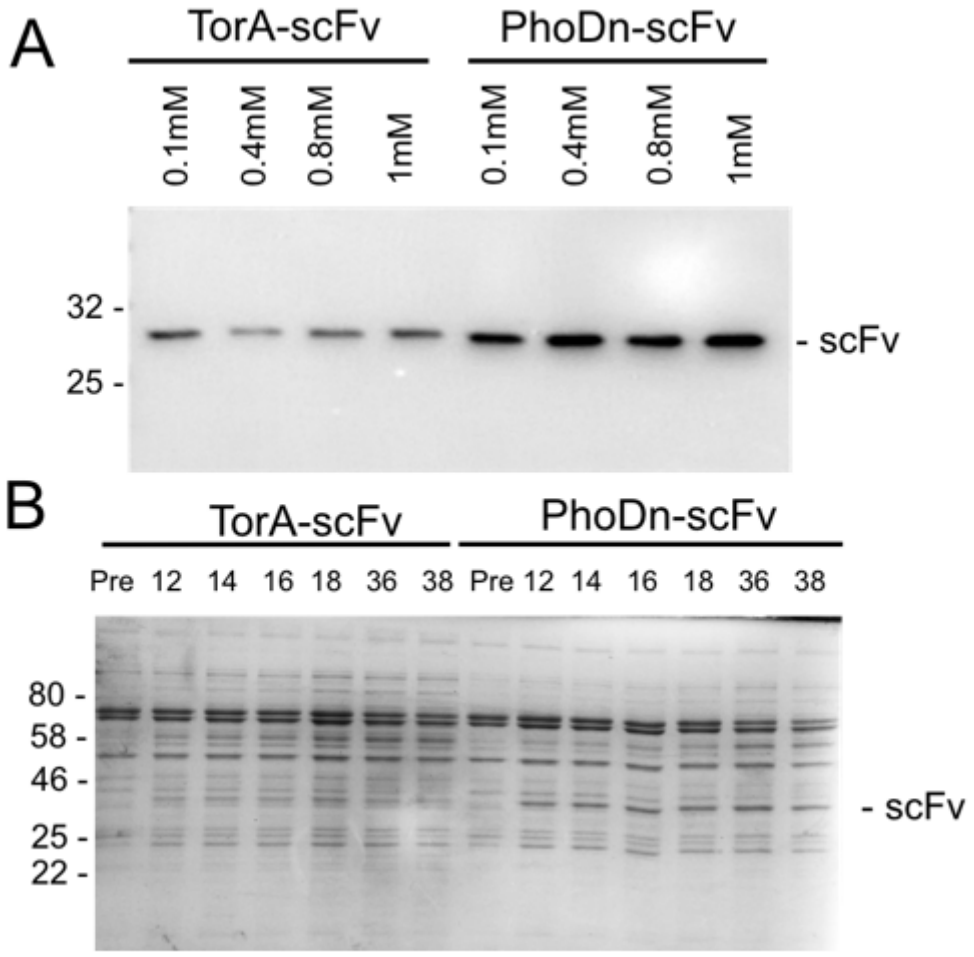

Figure 6

PhoDn-scFv is exported at significantly higher levels than TorA-scFv during fed-batch fermentation in the ambr250. A: comparison of the TorA-scFv and PhoDn-scFv levels exported at the 4 different IPTG concentrations as shown in Figure 5. Samples of the 38 h post-induction samples were analysed by immunoblotting. B: samples from the TorA-scFv and PhoDn-scFv cultures induced using 1.0 mM IPTG, taken at various time points (in h) were run on the same gel to assess periplasmic scFv levels. Pre: pre-induction sample.