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Approaches for high-throughput quantification of periplasmic recombinant proteins

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ABSTRACT

The Gram-negative periplasm is a convenient location for the accumulation of many recombinant proteins including biopharmaceutical products. It is the site of disulphide bond formation, required by some proteins (such as antibody fragments) for correct folding and function. It also permits simpler protein release and downstream processing than cytoplasmic accumulation. As such, targeting of recombinant proteins to the *E. coli* periplasm is a key strategy in biologic manufacture. However, expression and translocation of each recombinant protein requires optimisation including selection of the best signal peptide and growth and production conditions. Traditional methods require separation and analysis of protein compositions of periplasmic and cytoplasmic fractions, a time- and labour-intensive method that is difficult to parallelise. Therefore, approaches for high throughput quantification of periplasmic protein accumulation offer advantages in rapid process development.

Recombinant protein production in *E. coli*

Recombinant protein production (RPP) involves the overexpression of a protein of interest, usually in an organism different to the native origin of the protein. The overriding goal is the eventual purification of a high quantity of biologically active protein product. RPP has developed into a multi-billion-pound industry, responsible for the large-scale production of proteins used for research, diagnostics, and biotherapeutics [1]. Several different manufacturing hosts are available for RPP; aligning the properties of a recombinant protein with that of the production host is an essential step in designing a robust process. In the manufacture of biotherapeutics, which constitutes the largest revenue stream for RPP, mammalian hosts such as CHO cells are the most frequently used as they can perform the complex post-translational modifications for synthesis of active human proteins such as monoclonal antibodies [1]. However, the complexity, slow growth, and medium requirements of CHO cells means that such processes are expensive.

If the recombinant protein of interest requires minimal post translational modifications, it is often manufactured in *Escherichia coli*, a host second only to CHO cells for the manufacture of biotherapeutics [1] and a common host for manufacture of proteins for diagnostic and research purposes [2]. Biotherapeutics synthesised in *E. coli* include insulin, colony stimulating factors, hormones, cytokines, growth factors and antibody fragments [1]. *E. coli* is advantageous as it has simple nutritional requirements, fast growth kinetics, can be grown to high cell densities and thus a high potential titre of recombinant protein, and has a long history of safe use and process developmental experience.

While *E. coli* is does not possess the necessary pathways for many complex post-translational modifications such as N-linked glycosylation, disulphide bond formation is possible, mediated by the endogenous Dsb disulphide bond formation system [3] (we direct readers to [4] for an explanation of the function of the Dsb system). It is therefore possible

Abbreviations: APEx, Anchored Periplasmic Expression; Bla, Beta lactamase; CHO, Chinese Hamster Ovary; CYB5, Cytochrome b_5; Dsb, Disulphide bond formation; ELISA, Enzyme-linked immunosorbent assay; Fab, Antigen binding fragment; FACS, Fluorescence-activated cell sorting; FDA, US Food and Drug Administration; FlAsH, Fluorescein arsenical hairpin binder; FRET, Förster resonance energy transfer; GFP, Green fluorescent protein; IgG, Immunoglobulin G; MAP, methionine aminopeptidase; MBP, Maltose binding protein; MIC, Minimum inhibitory concentration; NLR, nanolitre reactor; PECS, Periplasmic expression with cytometric screening; pPACE, periplasmic phage-assisted continuous evolution; PPIase, cis-trans peptidyl-prolyl isomerase; RPP, Recombinant protein production; scFv, Single chain variable fragment; sfGFP, Superfolder green fluorescent protein; SPaseI, Signal peptidase I; SRP, Signal recognition particle; Tat, Twin arginine translocation; TEV, Tobacco etch virus; TIR, translation initiation region; TMR, carboxytetramethylrhodamine; WT, Wild type; XP, 5-bromo-4-chloro-3-indolyl phosphate; YFP, Yellow fluorescent protein.

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to synthesise proteins like antibody fragments such as Fab and single chain variable fragments (scFvs) where lack of glycosylation does not impair in vivo function [5]. The biopharmaceutical antibody-based products made in *E. coli* at the time of writing are [1]: Lucentis® (IgG fragment, Novartis / Genentech, FDA approval 2006); Cimzia® (Fab' fragment, UCB Pharma, 2008); Cablivi® (V_{HH} nanobody, Ablynx / Genzyme, 2019); Susvimo™ (IgG1κ antibody fragment, Genentech, 2021); and Byooviz™ (Biosimilar to Lucentis, Samsung Bioepis NL / Biogen, 2021). The recent spate of FDA approvals potentially demonstrates the resurgence of *E. coli* as a host for antibody fragment production.

In *E. coli*, the Dsb system is localised to the periplasm [3], and so targeting of recombinant proteins to this extra-cytoplasmic compartment is desirable. Accumulation of recombinant protein in the periplasmic space has several additional advantages compared with cytoplasmic expression. Increased product solubility and simpler purification is possible, as the periplasm is less protein-dense than the cytoplasm [6]. Product stability may improve due to lower protease activity in the periplasm [7]. Several cis-trans peptidyl-prolyl isomerases (PPIases) are present in the periplasm [8], which can improve biological activity of a product. Finally, removal of the N-terminal methionine can be essential for activity or prevent immunogenicity of eukaryotic proteins. Cleavage of a signal peptide during export to the periplasm subverts this problem [9], although it should be noted that N-terminal methionine removal can also occur cytoplasmically via the enzyme methionine aminopeptidase (MAP) [10].

Synthesis of heterologous protein in the *E. coli* periplasm is not always straightforward, particularly if the recombinant protein is very large or has multiple domains. Indeed, great efforts are being taken to increase periplasmic yields of recombinant proteins, as recently reviewed by [11]. Overexpression of any protein, irrespective of the sub-cellular compartment it is directed to, can promote undesirable cellular responses. Partitioning of resources (metabolites and energy) can cause conflict between biomass and protein generation, commonly referred to as metabolic burden. Expression vectors are often designed to ensure maximum accumulation of a recombinant protein, by way of strong promoters and high copy number, which can exacerbate metabolic burden. Misfolding of recombinant proteins can trigger heat shock responses and lead to inclusion body formation [12]. Whereas translation and folding appear to be the limiting steps for expression of cytoplasmic proteins, saturation of the translocation machinery is believed to be the major bottle neck when localising a recombinant protein to the periplasm [13,14].

Translocation of proteins to the periplasm – Sec and Tat

Proteins targeted to the periplasm in *E. coli* are translocated across the inner membrane by one of two pathways; Sec [15] or Tat [16] (Fig. 1). Sec-mediated export can be further split into SecA-dependent post-translational, or SRP-dependent co-translational branches, differing by whether export is coupled to translation. The Sec apparatus translocates polypeptide chains in an unfolded state, such that protein folding occurs in the periplasm, whereas Tat translocates proteins that have folded in the cytoplasm. In each case, an N-terminal signal peptide directs the polypeptide chain to the correct translocation apparatus.

The ubiquitous Sec machinery (Fig. 1A&B) is responsible for export of approximately 98% of *E.coli* proteins to the cell envelope [17]. Central to the Sec system is a heterotrimeric complex of integral membrane proteins: SecY [18]; SecE [19]; and SecG [20]. The hourglass shaped SecY forms the protein conducting channel, and its clamshell conformation forms a lateral gate opening into the bilayer, for insertion of proteins directly into the membrane [21]. SecE is also essential and stabilises SecY, preventing degradation by the protease FtsH [22]. SecG is nonessential but may improve ATP mediated translocon activity under certain conditions [20]. Two mechanisms direct polypeptides to SecYEG, each mediated by distinct targeting factors. The post-translational branch (Fig. 1A), driven by the ATPase SecA [23], translocates fully translated polypetide chains. The co-translational route (Fig. 1B), driven by SRP [24] begins translocation whilst the polypeptide chain is still being translated and is emerging from the ribosome. Generally, soluble periplasmic proteins and outer membrane proteins are exported by the post-translational branch; while inner membrane proteins, or those that fold quickly are exported co-translationally [15]. Both routes through Sec initiate interaction with the polypeptide during translation [25,26]. Early interaction of signal peptides with the Sec translocon presumably prevents folding of mature regions, or hydrophobic stretches aggregating in post-translational substrates. Similarly, for co-translational substrates, protein synthesis must be coupled to export to ensure translocation proceeds correctly.

The Tat pathway (Fig. 1C) is the alternative route by which proteins can be transported across the inner membrane, and unlike Sec, folded proteins are translocated by Tat [16]. Many Tat-dependent proteins are involved in redox reactions, necessitating folding around cytoplasmically-generated cofactors such as iron-sulphur clusters or molybdopterin guanine dinucleotide for activity [27,28]. Presently, is estimated that there are just 27 Tat-dependent substrates in *E. coli* [29]. While native substrates fold in the cytoplasm, Tat has some capacity for

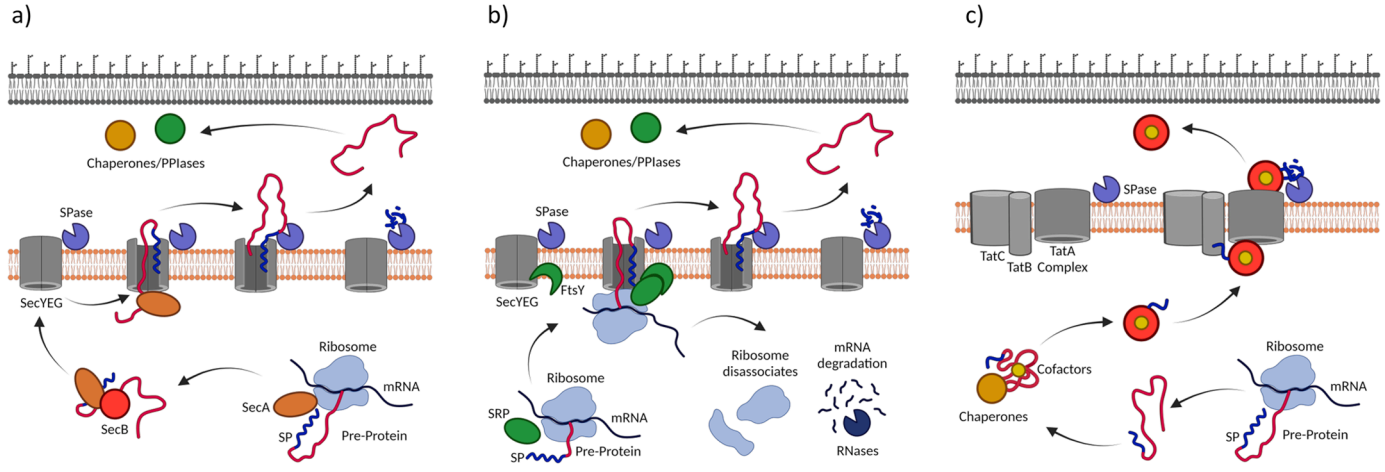

Fig. 1. Summary of Sec- and Tat-dependent translocation pathways. The SecYEG translocon is responsible for translocation of unfolded polypeptide chains via the SecA-dependent post-translational (a) and SRP-co-translational (b) pathways. Proteins that have folded in the cytoplasm are translocated through the Tat pathway (c). In each case, the signal peptidase (SPase) cleaves the signal peptide at the N terminal of the translocated protein (SP, shown in blue). Created with BioRender.com.

export of unfolded preproteins, provided they are of limited hydrophobicity and less than approximately 100 amino acids in length [29–31]. Three inner membrane proteins (TatABC) are central to Tat mediated export; TatB and TatC form complexes with one another and are believed to be the docking sites for signal peptides to start translocation [29,32]. TatC is very large, with six transmembrane domains, while TatB has a single transmembrane and a large cytoplasmic domain [33]. Binding of targets to TatBC induces the proton motive force (PMF) dependent association of TatA to TatBC [34]. Upon binding TatBC, the usually tetrameric TatA has a propensity to form higher order oligomers [35], and likely forms the pore through which substrates pass [36]. Unlike Sec, Tat export is ATP-independent and the PMF is used to drive substrates across the inner membrane [37].

Signal peptides mediate translocation

The route across the inner membrane is determined by properties of cleavable N-terminal signal peptides which initiate translocation through interactions with various export factors in the early stages of translation, as with Sec, or after folding, in the case of Tat. Signal peptides contain three common elements; the n-, h- and c-regions, each of which have a specific function, reviewed by [38]. The n-region typically has a net positive charge, the h-region is hydrophobic and has an α-helical conformation, and the c-region has a β-sheet confirmation and contains the SPaseI cleavage sites [39,40]. In addition to serving a targeting function, Sec-dependent signal peptides delay folding, often via non-optimal codons to induce a translational pause [41]. Both Sec branches have preference for more hydrophobic signal peptides [42]. Extreme hydrophobicity increases affinity to SRP, diverting proteins to the co-translational branch of Sec [43]. Conversely, glycine residues in the h-region interrupt the helical signal peptide conformation favoured by SRP, targeting the protein to the post-translational pathway [44]. Pathway switching has been demonstrated by increasing the hydrophobicity of the normally SecA-dependent Maltose Binding Protein (MBP) signal peptide and switching it to SRP-dependence [43].

Tat-dependent signal peptides work slightly differently, the targeting components of the system, TatB and TatC, are inner membrane localised, while the preprotein will have attained tertiary conformation prior to translocation [16]. Tat-dependent signal peptides contain an h-region that is, on average, less hydrophobic than in Sec-dependent signal peptides, and have a longer n-region [40]. Between the n and h regions is a unique Ser/Thr-Arg-Arg-X-Phe-Leu-Lys consensus motif, the two sequential arginine residues being responsible for the name of the pathway, twin arginine translocation [45]. The importance of the Lys residue at the C-terminus of the motif is ambiguous [46], and this residue is missing from some descriptions of the consensus motif [47].

Considerable effort has been directed toward correlation of signal peptide sequence and functionality in terms of export of recombinant proteins [40]. Rational design of a signal peptide to improve secretion has been attempted, for example insertion of a basic residue at the second position from the N-terminus [48], increasing the hydrophobicity of the h-region [49] or improving the efficiency of SPaseI cleavage [39]. However, signal peptide function with different recombinant proteins is wildly unpredictable [50,51]. There is no guarantee a signal peptide that permits effective production and translocation of one recombinant protein will do so for others.

While essential for translocation, signal peptide selection also inadvertently influences translation initiation [52], a rate limiting step during protein synthesis. The translation initiation region extends from the 5' untranslated region upstream of the Shine-Dalgarno sequence to around the first five codons on the mRNA [53]. Translation begins with the 30 S ribosomal subunit binding fMet-tRNA, supported by initiation factors and subsequent complex with the initiation region on the mRNA [54]. Fusing a signal peptide to the N-terminus of a recombinant protein modifies this initiation region around which the ribosome assembles for translation. This may be non-optimal and affect interaction with the ribosome, or alter mRNA secondary structure [55,56].

The requirement for screening approaches for periplasmic production

Localising a given protein to the *E. coli* periplasm has no generalisable strategy – what works for one protein may be ineffective for another. Multiple factors can determine accumulation of a recombinant protein in the periplasm. Physical parameters such as temperature can be used to slow down expression, while growth media is optimised to provide a metabolic surplus. Design of the expression construct is also essential. Like all expression systems, the choice of promoter must be considered. Stronger promoters drive greater gene expression, but at the expense of increased stress placed upon the host. Inducible transcriptional control is required to precisely control the point at which RPP begins, allowing coupling or de-coupling to biomass accumulation. Systems are available which can precisely tune expression levels using titratable promoters, for example the rhamnose-induced P^{rhaBAD} [57,58], or incorporate riboswitches for additional control over translation rates [59]. Tuning this way can allow for better balance between the rates of transcription, translation and translocation, minimising bottlenecks (for example accumulation of polypeptide chain in the cytoplasm prior to translocation, which can lead to misfolding [58].

The number of factors influencing export and the inherent unpredictability in how a given protein will accumulate in the periplasm necessitates systematic screening. Typically, accumulation of a recombinant protein in the periplasm is measured by harvesting cells, separating them into periplasmic and spheroplast fractions (for example using osmotic shock) followed by analysis of protein content of fractions using SDS-PAGE, ELISA or Western blotting. Disulphide bonding can be analysed by comparison of reducing- and non-reducing SDS-PAGE (for example [58]). These are slow and laborious workflows which severely hamper the throughput of the optimisation process. Methods which allow for the high throughput assaying and quantification of recombinant protein accumulated in the periplasm are therefore desirable for strain and process development [60]. Ideally, such a screen would permit simple, rapid and parallelisable measurement for example by spectroscopic, fluorimetric or luminescence measurements. In this review we will summarise some of the more recent methods for the rapid quantification of periplasmic accumulation of recombinant proteins in *E. coli*.

Fig. 2 summarises these approaches, which rely upon fusion of a tag to the recombinant protein of interest. This tag might be directly detectable (for example by fluorescence), have a measurable enzymatic activity, or rely upon interaction (either covalent or noncovalent) with a fluorescent partner for detection.

Fluorescent proteins

A wide assortment of autofluorescent proteins are available, with many examples of their use as reporters on processes including gene

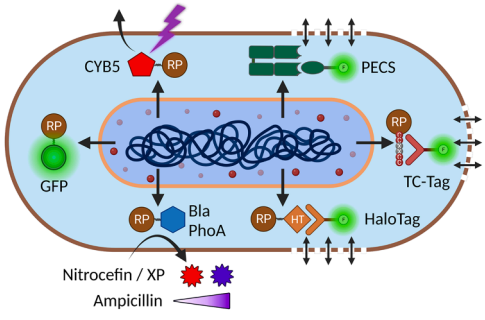

Fig. 2. Summary of periplasmic screening methods. Different methods for rapid detection of periplasmic protein concentrations as described in the text. Created with BioRender.com.

expression [61], protein folding [62], accumulation [63] and purification [64]. For a more thorough review of general applicability to RPP, readers are directed to [65]. The original and most frequently used is green fluorescent protein (GFP), a 26.9 kDa derivative of the AvGFP isolated from the jellyfish *Aequorea victoria* [66]. It has a distinctive β-barrel conformation with the tripeptide fluorophore Ser-65 – Tyr-66 – Gly-67 localised at the centre [67,68]. GFP fluorescence requires no ATP or cofactor, although oxygen is needed for fluorophore formation and fluorescence [69]. Wild type GFP has an excitation maximum of 395 nm, with maximum emission at 509 nm, permitting measurement using a fluorimeter, plate reader, or flow cytometer. Many derivatives of GFP have been created, thoroughly reviewed by [70]. Examples include altering the absorption maximum to a single peak (typically blue-shifted to around 488 nm), while maintaining the same emission wavelength

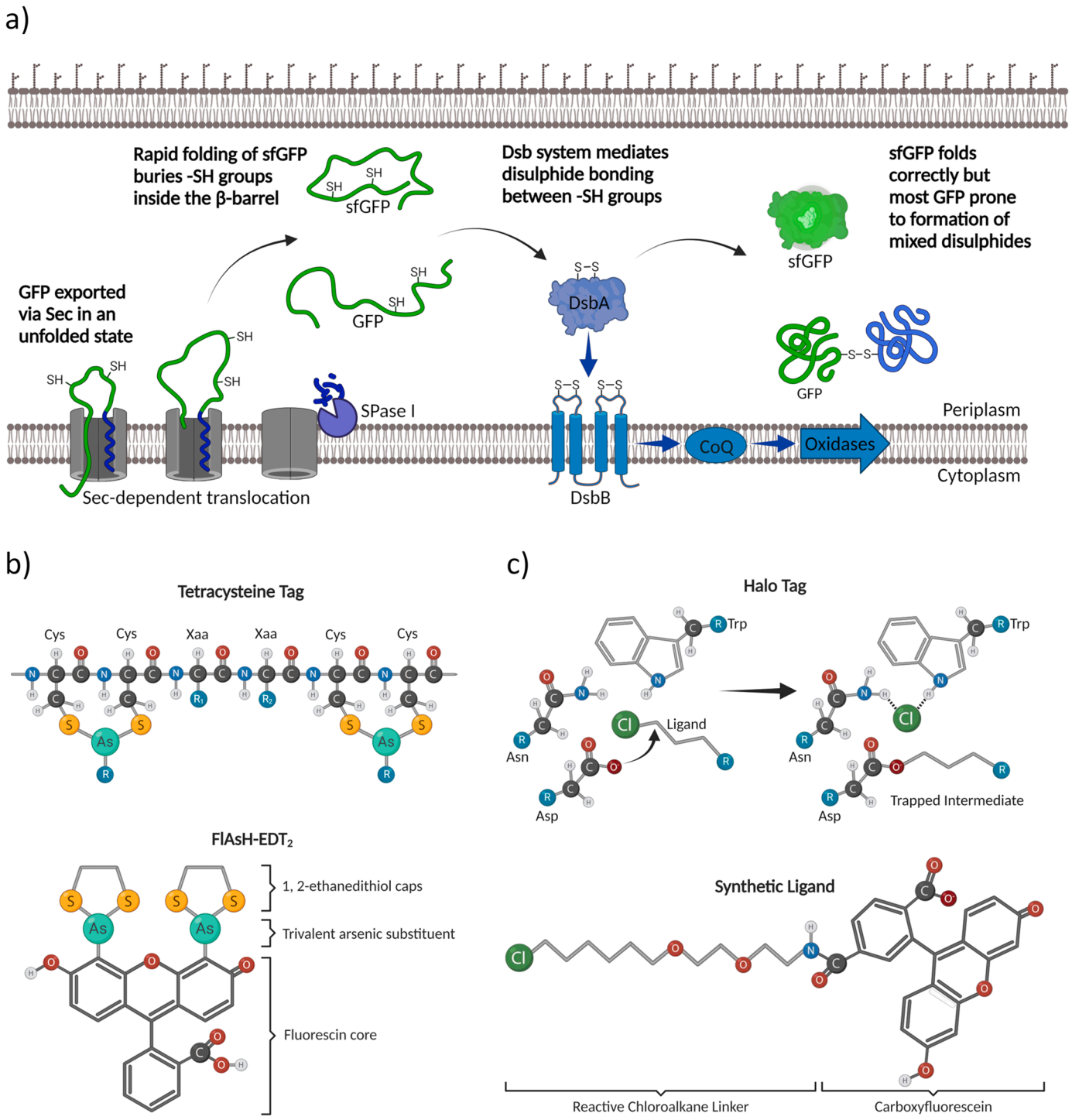

Fig. 3. Fluorescence based periplasmic screening methods. Fluorescence-based reporters permit access to powerful cytometric techniques for rapid screening. Fluorescent proteins are often utilised, although most GFP derivatives misfold upon export to the periplasm due the Dsb system (a), sfGFP folds rapidly circumventing this. Alternatives include ligand-based systems. Short peptides like the tetracysteine tag (b) exploit binding between arsenical compounds and paired thiol groups of cysteine residues. Halo tags (c) utilise a modified dehalogenase which covalently traps a fluorescently labelled ligand within its active site. Created with BioRender.com.

[71]; those improving folding at 37°C as with the cycle 3 mutations [72]; increasing brightness such as enhanced GFP (EGFP) [73]; or monomeric variants which do not oligomerise to form quaternary structures [74]. Other alterations shift the absorption and emission wavelengths, to produce blue, cyan and yellow fluorescence emission [68,75].

Fusion of GFP to the C-terminus of a cytoplasmically-targeted recombinant protein permits quantification of accumulation and also determination of folding state, as correctly-folded RP tends to correlate with correctly-folded GFP and thus higher fluorescence [76,77]. Fluorescence can be measured by fluorimetry, microscopy, or flow cytometry; the latter technique offers the added potential of identifying subpopulations [77]. However, GFP is not a straightforward reporter for periplasmic protein production as most GFP derivatives cannot correctly fold and fluoresce in the oxidising periplasm (Fig. 3A) [78]. GFP has two cysteine residues, C48 and C70, residing on the interior of the β-barrel [79]. If the protein is exported to the periplasm in an unfolded conformation, as required by the Sec export pathway, these residues are exposed to the oxidative Dsb system. Aberrant disulphide bonding, either between GFP molecules or with an independent partner, is theorised to prevent correct folding of the β-barrel essential for fluorescence [80].

It was predicted that a GFP variant which folds quickly could reach a mature state in the periplasm prior to being confined to non-fluorescence. One such variant, superfolder GFP (sfGFP) was isolated in a screen for more robust folding [81]. sfGFP can fold correctly and fluoresce even when fused to a misfolded partner, while folding kinetics are greatly improved. However, when sfGFP was fused to the DsbA signal peptide ($DsbA^{SP}$) or Maltose Binding Protein signal peptide (MBP^{SP}), thought to be exported via co-translational and post-translational pathways respectively, most fluorescent protein accumulated in the cytoplasm, with a little non-fluorescent product in the periplasm [82]. It was subsequently shown that sfGFP can indeed fluoresce in the periplasm; when using a full length MBP::sfGFP fusion with its own signal peptide [83] or fused to a mutant co-translational MBP^{SP*1} signal peptide [84]. There were two explanations offered for this discrepancy: first, expression strength, as sfGFP fluorescence in the periplasm was achieved using chromosomally-encoded sfGFP (single copy) whereas the high copy number plasmid used by [82] resulted in a lack of periplasmic fluorescence.

Second, authors suggested during post-translational export, fusion to just the MBP signal peptide may block SecYEG. Shorter fusions reduce the time between Sec recognition at the ribosome [25,26] and engaging the Sec translocon at the membrane. This compounded by faster folding kinetics of sfGFP, was proposed to lead to blockage. Therefore, fusions to full length proteins are more likely to be exported efficiently using the post-translational branch of Sec. In support of this, it was shown a $DsbA^{SP}$::sfGFP fusion, thought at the time to be exported co-translationally, was much more fluorescent in the periplasm than MBP^{SP}::sfGFP [83]. Furthermore, the MBP^{SP*1} signal peptide with three point mutations, thought to switch export to the co-translational pathway [43], produced fluorescently active sfGFP in the periplasm [84]. Therefore, either co-translational export or fusions to longer proteins appears critical for accumulation of active sfGFP in the periplasm. A recent study [85] has used a comparable approach with the mTurquiose2 protein as a FRET donor, introducing superfolder mutations to enhance folding to generate sfTq2; periplasmic fluorescence was achieved using the $DsbA^{SP}$.

It should be noted that more recent work that used selective ribosome profiling to determine the binding partners of SRP within the *E. coli* proteome [86] found that $DsbA^{SP}$ does not associate with SRP. The initial study that designated $DsbA^{SP}$ as directing co-translational translocation [87] pointed out that direct $DsbA^{SP}$-SRP interaction had not been demonstrated, and discussed the technical difficulty of proving conclusively whether a signal peptide directed co- or post-translational translocation. Signal peptides could direct proteins via both pathways, and studies with knockouts in *ffh* (encoding the protein component of SRP) can generate ambiguous data. These findings demonstrate that distinction between co- and post-translational pathways is still not fully understood, and that data should be interpreted carefully. While most GFP variants are poorly fluorescent in the periplasm, other fluorescent proteins have been shown to reach an active conformation. DsRed isolated from *Discosoma* [88] is devoid of cysteine residues. A monomeric derivative mRFP1 has been shown to fluoresce in the periplasm when fused to the extra-cytoplasmic C-terminus of the inner membrane protease YaeL [89]. However, mRFP1 matures much more slowly and is less fluorescent than GFP [90]. More recent DsRed derivatives are far brighter and mature more quickly; the widely used mCherry has been shown to fluoresce in the periplasm, regardless of which Sec pathway is chosen [83].

Other attempts have been made to specifically modify GFP variants to mature in oxidising environments. Site directed mutagenesis was used to change the C48 and C70 residues within the EGFP variant sGFP2, which already has improved folding and fluorescence in bacteria [91]. Mutagenesis of the two cysteine residues (C48S and C70M) created the cysteine free variant cfSGFP2 [92], which displayed remarkably similar photochemical properties to sGFP2 with only a slight shift in the excitation maximum from 497 to 493 nm. It was shown cfSGFP2 had increased fluorescence when exported to the Golgi apparatus (also an oxidative environment) in monkey-derived COS7 cells [92]. It is highly possible that cfSGFP2 would also fluoresce in the bacterial periplasm. More recently, a sfGFP derivative with C48S, C70S and a monomerising mutation, moxGFP, was shown to fluoresce in the periplasm [93]. Cysteine residues have also been removed from the sfTq2 protein, leading to enhanced periplasmic fluorescence and lower toxicity [85].

GFP derivatives that are non-fluorescent when exported through Sec can be rerouted through the Tat export pathway to ensure activity: for example, GFPmut3* was successfully translocated by fusion to the Tat-dependent $TorA^{SP}$ [94]. As the Tat pathway permits export of cytoplasmically folded substrates, GFPmut3* can reach an active conformation prior to export. Similarly, YFP, a red-shifted GFP derivate [68], is poorly fluorescent in the periplasm when exported via Sec, but active when exported via Tat [89]. Once folded, GFP is very stable and the location of the cysteines on the β-barrel interior means they cannot be accessed.

There are several additional points to consider when designing a recombinant protein-fluorescent protein fusion. The fluorescent protein must not sterically interfere with the recombinant protein, so to ensure correct folding of each. A flexible linker composed of small polar amino acids like glycine, serine or threonine is generally inserted between fusion partners, thoroughly reviewed by [95]. If functional studies of the recombinant protein are desired following synthesis, a protease site such as for TEV protease can be inserted up- or downstream of the linker, for cleavage in vitro [96]. Many fluorescent proteins are known to exhibit quaternary structure, particularly at high concentrations, which may interfere with fusion partner folding. Such oligomerisation should be avoided by using monomeric derivatives (in the case of GFP derivatives, those with the A206K mutation [74]).

In addition, some optimisation is required; while fluorescence is generally indicative of correct folding of both fusion partners, it does not immediately confirm localisation to the correct compartment. Fusions targeted for export may become trapped in the cytoplasm and still fold correctly. This is particularly important in RPP as overexpression can quickly saturate translocation machinery, leading to a secretion-deficient state [13,14]. A simple way around this is use of fluorescence microscopy to identify fluorescent halos around the periphery of the *E. coli* cells [94]. This could be automated by using image analysis or imaging flow cytometry.

Self-labelling tags: PECS and FlAsH tags

Periplasmic expression with cytometric screening (PECS) [97] is a fluorescence-based method for screening protein libraries for binding

affinity, which can be modified for use in monitoring periplasmic protein expression. PECS was initially developed as an alternative to protein surface display technologies in which libraries of genes of interest are fused to peptides directing them to the surface of the host organism (most frequently phage, bacteria or yeast [98]. Surface presented proteins are subsequently screened for their ligand binding properties. Surface display presents several disadvantages: fusion to a surface directed polypeptide may inhibit protein function; surface display might negatively impact the physiology of the host; or the proteins may be unsuited to surface presentation. In PECS, proteins of interest are periplasmically expressed via fusion to N-terminal signal peptides, so interference is minimal compared to larger surface display-mediating polypeptides. Bacteria are then subjected to outer membrane permeabilisation via incubation in high-osmolarity buffer, treated with a fluorescently-labelled ligand of the protein of interest and washed to remove unbound conjugate. Fluorescence is thus coupled to protein-ligand interaction and clones presenting desirable binding isolated via fluorescence-activated cell sorting (FACS). This method has been used to isolate scFvs with high affinity to digoxigenin [97].

While originally intended for screening combinatorial libraries, PECS has been re-purposed for use in expression studies, where the desired trait is not enhanced protein-ligand interaction, but rather periplasmic localisation of a protein [99]. In this case, PECS was used to optimise expression of full length aglycosylated IgG in *E. coli*, to improve yield in shake flasks, a process scale which is vital during initial screening of antibody variants following combinatorial library screening. Factors mediating high periplasmic accumulation of the IgG such as promoter, signal peptides for heavy and light chains, translation initiation region (TIR) and co-expression of chaperones were assessed. Following PECS-assisted optimisation, active IgG could be synthesised in the periplasm at levels 6-fold greater compared with WT *E. coli*.

PECS is a ligand binding-based method, so relies upon production of a protein which binds to a known ligand which can be fluorescently labelled; hence its use with antibodies and their fragments. For many recombinant proteins, binding partners may be undefined or unknown, or their interaction may be too weak or nonspecific. In these cases, alternative cytometric methods substituting the fluorescently-conjugated antigen for a peptide tag can be used. Fluorescein arsenical hairpin binder-ethanedithiol (FlAsH-EDT_2) is a tag which selectively binds the tetracysteine motif Cys-Cys-X-X-Cys-Cys, upon which it fluoresces (Fig. 3B) [100]. The protein of interest is directed to the periplasm via an N-terminal signal peptide, with the tetracysteine motif fused to the C-terminus. Similar to the PECS protocol, the OM is permeabilised by incubation of cells in salt-containing buffer followed by addition of FlAsH- EDT_2 and washing away any unbound tag. Higher accumulation of the recombinant protein to the periplasm produces greater fluorescence (λ_{ex} = 508 nm, λ_{em} = 528 nm) using FACS. An alternative to FlAsH is a red biarsenical dye, ReAsH, (λ_{ex} = 592 nm, λ_{em} = 606 nm) [101].

PECS-FlAsH screening, combined with Tn5 transposon insertion mutagenesis has been utilised to identify clones with improved SRP-mediated export of a model $DsbA^{SP}$-MBP model fusion [102]. Six SRP-enhanced mutants were isolated by fluorescence intensity and increased accumulation confirmed by SDS-PAGE. Interestingly, each had disruption at the same locus – the 16 S ribosomal RNA gene, *rrsE*. Export of MBP using its native signal peptide did not result in additional periplasmic accumulation, indicating the *rrsE* knockout is specific to the SRP pathway. Disruption of the *rrsE* gene in hosts overexpressing endogenous SRP-dependent proteins DsbA, TolB and TorT also led to increased periplasmic accumulation compared to the parental MG1655 strain. The *rrsE* knockout was also shown to increase periplasmic accumulation of mammalian proteins M18 scFv (3.1-fold increase), full length IgG (2.7-fold increase) and NTR1 G protein-coupled receptor (2.6-fold increase). While the exact mechanism by which *rrsE* knockout improves SRP-dependent export is unclear, its discovery further highlights the benefit of PECS-FlAsH in not only screening known factors affecting periplasmic localisation, but also identifying new ones.

PECS-FlAsH presents other advantages over fluorescent reporter techniques such as GFP fusions. Tetracysteine motifs are just six amino acid residues in length, while more intensely fluorescent optimised variants are up to 12 [103]. These tags are therefore likely to have fewer effects on the translation, translocation and folding of a recombinant protein than GFP. Furthermore, unlike GFP variants which preferentially translocate through the SRP pathway, the FlAsH tag is compatible with both co- and post-translational pathways, permitting screening of both routes. It should also be noted that FlAsH tags have been used to quantify and optimise extracellular protein production, for example via the native YebF pathway and heterologous type III secretion system [104].

Self-labelling tags: HaloTags

The HaloTag (HT7) is another variant of self-labelling tag, and like the tetracysteine motif, has a number of advantages over fluorescent proteins for monitoring periplasmic accumulation (Fig. 3C) [105]. The HaloTag is derived from the haloalkane dehalogenase (DhaA) enzyme of *Rhodococcus rhodochorus* which catalyses the removal of halogen atoms from haloalkanes via cleavage of the halogen-carbon bond. The HaloTag was designed to improve upon the capabilities of other non-fluorescent fusion tags, namely a tag-ligand interaction whose binding kinetics are rapid, selective and irreversible [106]. DhaA is just 34 kDa in size and monomeric, so is a less intrusive fusion than some other fusion proteins. It also has a broad substrate specificity, increasing the number of potential ligands [107,108]. Further, the authors indicate the haloalkane substrates are chemically simple and membrane permeable.

During the reaction catalysed by the wild type DhaA enzyme, a reaction intermediate is formed whereby the dehalogenated alkane is covalently linked to DhaA. The alkane is liberated, and the enzyme regenerated via a mechanism involving residue H272. Mutation of H272 to phenylalanine results in a DhaA enzyme that can cleave the carbon-halogen bond but the resultant alkane is covalently trapped in the active site. Use of a fluorescently-labelled haloalkane results in covalent fluorescent labelling of the mutant DhaA enzyme [105,106]. Initial studies used the HaloTag with TMR (carboxytetramethylrhodamine) and FAM (carboxyfluorescein) fluorophores linked to chloroalkane linkers to image mammalian cells [105]. The initial HaloTag was optimised through mutagenesis to generate HT7 [106].

HT7 has been utilised to visualise proteins in the *E. coli* periplasm via a C-terminal fusion with DsbA [109]. Cells expressing the fusion can be inoculated onto solid growth media containing 5 nM of the TMR labelled haloalkane ligand and incubated at 30 °C. Fluorescence can then be detected (λ = 532 nm, λ_{em} = 580 nm). Cells could also be labelled in liquid culture. In both instances, cells expressing the DsbA::HT7 fusions were fluorescent, in addition to a $DsbA^{SP}$::HT7 fusion. Importantly, cells not expressing the HT7 tag were not fluorescent. When mixing HT7 + and HT7- strains in culture and plating, individual HT7 + colonies could be easily identified by their fluorescence. As cysteine residues in GFP are believed to result in misfolding, a strain expressing a HT7 tag with the two cysteines at positions 61 and 262 substituted for serine were tested. This variant had 3-fold less fluorescence than the original HT7. However, when fused to the cytoplasmic protease ClpP, there appeared to be similar levels of fluorescence, so it does not appear that HT7 tags suffer from misfolding when directed to the periplasm. Two areas still to consider would be firstly, whether the tag can be directed to the periplasm via the post-translational pathway, or if it folds too quickly for export. Secondly, a more high-throughput detection method would be beneficial, to facilitate screening for high accumulating cells in RPP optimisation. A fluorescent microtiter plate reader could be used, or alternatively flow cytometry analysis. HaloTags have already been used in combination with flow cytometry and human Jurkat cells to study infection by tagged HIV [110].

CYB5 Tags

While the above methods rely on fluorescence, other tags to monitor periplasmic accumulation directly detect their environment. Cytochrome b_5 (CYB5) is a ubiquitous haemprotein that can be utilised as a visual tag with spectrophotometric detection [111]. Unlike some other chromogenic proteins, CYB5 is a strong absorber of light in the blue region (A_{max} = 413 nm), while its molar attenuation coefficient (ε) is 117,000 $M^{-1}{\cdot}cm^{-1}$ providing it with an intense red colouration and therefore a small lower detection limit. CYB5 senses the oxidation state of its environment; its maximum absorbance shifts from A_{max} = 413 nm, with two very small peaks at 532 nm and 559 nm when oxidised, to A_{max} = 424 nm and two peaks at 527 and 557 nm when reduced [112]. Thus, periplasmic accumulation of a RP-CYB5 fusion can be detected by a shift in the absorbance spectra.

CYB5 also offers stability across a substantial pH range offering the potential for use throughout various steps in a bioprocess rather than just upstream process development. This approach was used to optimise periplasmic production of scFv and Fab antibody fragments, using Rat CYB5 as a C-terminal fusion [111]. Yield of the scFv increased almost three-fold when expressed in a fusion with CYB5, while a greater than two-fold increase was seen for the Fab-CYB5 fusions. This suggests that CYB5 could be used as an expression enhancer in addition to its spectral properties. CYB5 is extremely soluble (predicated at 95%) so is thought to facilitate improved folding of a fusion partner and enhance expression [113].

Enzyme tags: alkaline phosphatase and β-lactamase fusions

Enzymes which are active only in the periplasm (often due to the requirement for disulphide bonding) can be used as fusions to detect periplasmic localisation. The periplasmic *E. coli* alkaline phosphatase (encoded by *phoA*) has been used as a fusion for studying protein translocation into the periplasm [114]. PhoA activity was measured using the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP), and the requirement for a signal peptide (here, Bla^{SP}, $OmpF^{SP}$ and $LamB^{SP}$) for activity was demonstrated. Applications of PhoA fusions were subsequently reviewed [115]. One use of alkaline phosphatase is determination of membrane protein topology; use of PhoA (active in the periplasm) and β-galactosidase LacZ (active in the cytoplasm) fusions to different positions of a membrane protein permit mapping of transmembrane, cytoplasmic, and periplasmic portions of the protein [116]. It should be noted that the activity of PhoA in the cytoplasm increases over time in non-growing cells [117], which could generate erroneous results. Nonetheless, PhoA has been used to probe signal peptide structure-function relationships [118] and to characterise the region of the SecA protein that binds to signal peptides [119].

TEM-1 β-lactamase (Bla), which cleaves the β-lactam ring of antibiotics such as penicillin, is active in the periplasm and can be used as a fusion tag to report periplasmic protein accumulation. This approach has been used as an assay of protein folding in the periplasm, utilising the SRP-dependent DsbA signal peptide [120]; screening was based on determination of ampicillin resistance. Broad applicability was demonstrated by screening the periplasmic folding of a range of recombinant proteins, determining the effect of periplasmic chaperones, and the directed evolution of a peptide. A similar approach was also used to optimise the OmpA and PelB signal peptides for the export of Bla alone [121]; while screening signal peptides fused to Bla combined with a directed evolution approach, was used to optimise the translation initiation region created during cloning [122].

We have previously used Bla as a C-terminal fusion to screen for optimised signal peptides for periplasmic export of a scFv [123]. We measured periplasmic scFv-Bla accumulation via two functional assays: resistance to ampicillin, determined using a minimum inhibitory concentration (MIC) assay; and via the chromogenic β-lactam nitrocefin [124] which upon cleavage can be quantified by measurement of absorbance at 495 nm. Cytoplasmic Bla was shown not to confer resistance to ampicillin. Our general approach was to use libraries of signal peptides based on the PelB signal peptide [125] generated by error-prone PCR or chemical synthesis. These libraries were screened for periplasmic accumulation of a scFv-Bla fusion first by selection of colonies resistant to ampicillin, then by screening using nitrocefin. Isolates were then characterised after shake flask growth using periplasmic fractionation and SDS-PAGE.

We isolated mutated signal peptides in four functional categories; those giving rise to lower or similar periplasmic accumulation to the wild-type PelB signal peptide; those giving rise to higher translocation of scFv-Bla to the periplasm; and those where scFv-Bla production was higher than $PelB^{sp}$ but did not confer increased translocation to the periplasm. Highly translocating signal peptide variants had mutations that increased the positive charge of the n-region and altered hydrophobicity and / or were predicted to change the helical nature of the h-region, all proposed important for signal peptide function. In intensified fed-batch fermentations, periplasmic yields were higher for selected mutant signal peptides than the original PelB signal peptide, both for scFv-Bla fusions and after removal of the Bla fusion.

It was noted that for all signal peptides (original and mutated), specific periplasmic productivity for the scFv-Bla fusions were higher than that of the scFv alone, suggesting that the Bla fusion might improve translation, translocation or stability of the scFv. This is a potential hazard of using fusion tags; while aiding detection, the tag can alter the overall translation, folding, stability, or translocation of the protein of interest. It is therefore very important to validate the outputs of screens in the absence of the fusion tag. In addition, tags are only useful industrially during process development; cleavage of tags using proteases is not practical in production processes due to downstream issues. An industrial workflow would typically involve screening using a tag followed by validation of the screen outputs without the tag to select development candidates for production.

A comparable approach using Bla fusions was employed to select for mutations in the *E. coli* Tat apparatus enabling enhanced translocation, so-called “supersecreter” strains [126]. An scFv-Bla fusion was targeted to the periplasm via Tat using a TorA signal peptide. Bacteria also carried randomly mutagenized *tatABC* on a plasmid; mutants with high translocation were selected on the basis of elevated Carbenicillin resistance. Three such mutants were isolated, with mutations in *tatB* and / or *tatC* (although not *tatA*). Enhanced translocation of scFv without the Bla fusion was confirmed by Western blotting of subcellular fractions. Eight other proteins of biotechnological interest were also shown to be translocated more effectively by the selected strains, again using $TorA^{sp}$, Bla fusion and measurement of carbenicillin resistance. As each mutant strain contained multiple point mutations in *tatB* and / or *tatC*, a similar approach was used to identify the specific mutations responsible for translocation enhancement. Finally, it was found that each mutated Tat translocase exhibited suppression of quality control, leading to enhanced translocation. Overall, these studies demonstrate the promise of Bla fusions for screening, both for enhanced signal peptides and translocation apparatus.

Antibody fragments, affinity and display

Monoclonal antibodies represent the largest class of biopharmaceuticals, used to treat a range of cancers and other diseases such as rheumatoid arthritis [1]. Selection of antibodies on the basis of affinity to relevant antigens is a key part of the development of novel antibody therapeutics; because of the complexity of full-length monoclonal antibodies, antibody fragments such as scFv and Fab are frequently used as development tools [127]. Selection typically uses a display approach, where antibody fragments are displayed on the surface of virus particles or cells [128]. Mutagenesis is used to generate a library of genes encoding antibody fragments, so each cell displays an antibody fragment with different structure and thereby affinity. Cells are

incubated with a fluorescently-labelled antigen and fluorescence-activated cell sorting (FACS) is used to physically select cells displaying optimal fragments; the cell contains the relevant gene variant thereby permitting linkage of genotype and function. There are a number of such display approaches using *E. coli*, where antibody fragments are directed to the periplasm or cell surface, in each case requiring translocation across the inner membrane [129–131].

In the APEx (Anchored Periplasmic Expression) system [129], antibody fragments are fused either via the N-terminus to the leader peptide and first six amino acids of the inner membrane lipoprotein NlpA, or via the C-terminus to the M13 gene 3 minor coat protein. In order for the labelled antigen to access the antibody fragments in the periplasm, the outer membrane requires permeabilisation with EDTA and lysozyme. APEx was initially used to screen scFv libraries [129] and has since been used for screening full-length immunoglobulin Gs [132]. Translocation of antibody fragments through the outer membrane (permitting true surface display and screening without OM permeabilization) adds to the complexity of the approach, and for this reason *E. coli* surface display lags behind phage display and yeast surface display; readers are directed to [128] for a discussion of different display approaches.

A recent study reported a continuous selection platform for optimisation of protein-protein interactions in the periplasm, named periplasmic phage-assisted continuous evolution (pPACE; Fig. 4) [133]. This approach is a development of phage-assisted continuous evolution (PACE), a directed evolution method where M13 bacteriophage transfers genes between *E. coli* bacteria [134]. The infection rate of the phage is linked to the desired activity (e.g., protein-protein interaction) via control of expression of the pIII gene which mediates host cell entry. A major advantage of PACE is that it is continuous and does not require human intervention, permitting hundreds of rounds of directed evolution in a few days.

In the pPACE system, expression of gIII (controlling phage infection rate) is controlled by the CadC regulator. CadC spans the inner membrane and comprises a periplasmic sensor domain, a transmembrane helix, and a cytoplasmic DNA-binding domain. Native CadC dimerises when the periplasmic sensor domain detects high pH and low lysine concentrations. In pPACE, the periplasmic domain of CadC is replaced with an antigen. The system was used to engineer scFv fragments with high affinity to the leucine zipper GCN4, and novel scFv variants of the breast cancer therapeutic monoclonal antibody Trastuzumab to a Her2 mimetic peptide (Her2 being the therapeutic target of Trastuzumab).

Screening in Gram positive bacteria

Gram positive bacteria are not common hosts for production of biopharmaceuticals, although they are widely used for production of recombinant and native enzymes [135]. As they are bounded by a single membrane, translocation via Sec or Tat permits secretion into the culture broth. Many Gram-positive hosts for protein production permit effective secretion of products and as such, much research on the Sec translocon and signal peptides has focused on Gram positives [40]. Screening approaches here often rely upon secreted enzymes whose activity can be assayed in the culture broth.

Amylase activity can be measured using high-throughput assays; this reporter system has been used to Identify genes whose overexpression improves Sec-dependent translocation [136] and screen libraries of Sec-dependent signal peptides [137], both in *Bacillus subtilis*. Signal peptide library screening has also been completed using secreted lipolytic enzymes [138], protease [139], and cutinase [140]. A recent screen of signal peptides in *B. subtilis* used the secreted amylase AmyQ to hydrolyse a fluorescein-labelled starch [141]. Cells were grown in nanolitre reactors (NLRs), essentially microcompartmentalisation using

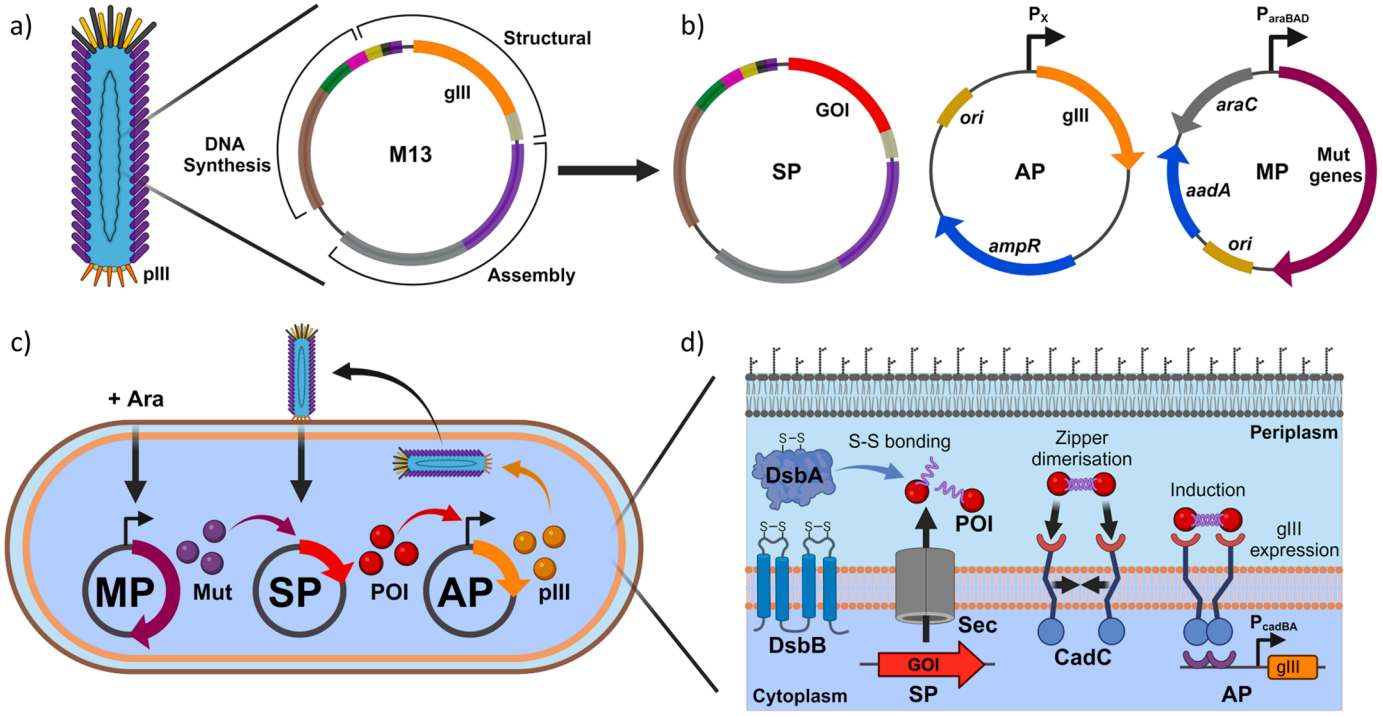

Fig. 4. The pPACE (periplasmic phage-assisted continuous evolution) system. pPACE is a development of PACE (phage-assisted continuous evolution), based on bacteriophage M13 (a). The product of gene III (gIII) is required for host cell entry. The PACE system (b) comprises a selection phage (SP) encoding the gene of interest (GOI) to be mutated and selected in place of gIII. The host cell also contains an accessory plasmid (AP) encoding gIII under the control of a promoter responding to the activity of the product of the gene of interest being mutated. An additional mutagenesis plasmid (MP) encodes genes that increase the error rate of DNA replication. In action (c), arabinose is used to induce mutagenesis from MP, the gene of interest is mutated, generating variability, and phage DNA containing the GOI is packaged into new phage particles. The phage infects new host cells; optimal GOI sequences give rise to enhanced infection rates. The mutagenic cycle takes 10 – 60 min. In pPACE (d), gIII expression is regulated by the dimerization of the periplasmic product of the gene of interest, inducing dimerization of the CadC transcription activator.

a water-in-oil emulsion, with each water droplet initially containing a single cell, and screened using FACS. Microcompartmentalisation approaches have similarly been used to screen for the production of a range of secreted compounds, as reviewed by [142]. If a comparable approach were to be used in *E. coli* or other Gram-negatives, the outer membrane would need to be permeabilised in some way to release the periplasmic proteins. However, envelope permeabilization would potentially risk leakage of cytoplasmic components across the inner membrane, invalidating the approach. Since envelope stability is compromised by stress, it would be difficult to set up such assays.

Conclusions

The theoretical advantages of periplasmic production of recombinant proteins in Gram-negative bacteria can be difficult to translate into real-world applications and processes as optimisation can be difficult. There are an expanding number of tools to assist development of such processes, although as can be seen, limitations are observed in most of these methods and these limitations must be considered when choosing experimental workflows.

Development of periplasmically-active fluorescent proteins continues, and these can be used for some screening work, although it is not clear how broadly applicable these are: for example, how do high concentrations of rapidly-folding fluorescent proteins in the *E. coli* periplasm affect bacterial physiology and native periplasmic proteins? Enzyme fusions show great promise although consideration must be given to how accurately enzyme activity reflects subcellular localisation. Both fluorescent protein and enzyme fusions could also alter the productivity, translocation, and folding of the protein of interest, requiring validation of results omitting the fusion. As with many branches of biotechnology, techniques must be used in combination to prevent erroneous conclusions from being drawn. Self-labelling tags represent a very useful tool as they function in a more orthologous manner than protein fusions; we expect that they will become more utilised in future. The pPACE method [133] represents a novel synthetic biology workflow of great potential commercial interest. Given the recent renewed commercial interest in antibody fragments as biopharmaceuticals, application of such approaches could accelerate future drug development.

As well as allowing optimisation of periplasmic RPP processes, these techniques have allowed better characterisation of the periplasmic translocation pathways, Sec and Tat. However, as has been seen, the exact mechanisms of translocation, and how the structure of the signal peptide interacts with the translocon (for example, whether $DsbA^{SP}$ directs peptides through the co- or post-translational pathways) are not fully understood. Tools to analyse periplasmic protein accumulation can therefore also be used to better understand the translocation pathways themselves.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alexander Osgerby reports financial support was provided by Biotechnology and Biological Sciences Research Council.

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