Development of a high yielding expression platform for the introduction of non-natural amino acids in protein sequences

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ABSTRACT
The ability to genetically encode non-natural amino acids (nnAAs) into proteins offers an expanded tool set for protein engineering. nnAAs containing unique functional moieties have enabled the study of post-translational modifications, protein interactions, and protein folding. In addition, nnAAs have been developed that enable a variety of bioorthogonal conjugation chemistries that allow precise and efficient protein conjugations. These are being studied to create the next generation of antibody-drug conjugates that combine improved efficacy, potency, and stability for the treatment of cancer. However, the efficiency of nnAA incorporation, and the productive yields of cell-based expression systems, have limited the utility and widespread use of this technology. We developed a process to isolate stable cell lines expressing a pyrrolysyl-tRNA synthetase/tRNApyl pair capable of efficient nnAA incorporation. Two different platform cell lines generated by these methods were used to produce IgG-expressing cell lines with normalized antibody titers of 3 g/L using continuous perfusion. We show that the antibodies produced by these platform cells contain the nnAA functionality that enables facile conjugations. Characterization of these highly active and robust platform hosts identified key parameters that affect nnAA incorporation efficiency. These highly efficient host platforms may help overcome the expression challenges that have impeded the developability of this technology for manufacturing proteins with nnAAs and represents an important step in expanding its utility.

Introduction

Over the past two decades, protein therapeutics have emerged as potent drugs to combat human disease. Their efficacy depends on the intrinsic specificity of proteins for their targets, as well as their mode of action, but many bioactive proteins have limitations that affect their half-life and potency, and thus their overall efficacy. With increased understanding of their function, protein engineering has enabled the construction of modified proteins to address these limitations through conjugations (e.g., pegylation) or by exploiting natural mechanisms for protein retention by half-life extension. Bi- and tri-specific antibodies represent bioengineered molecules that display novel functionality by enabling the binding of two or more antigens. Such molecules have been used, for example, for immune-oncology applications by simultaneously targeting a patient’s natural cytotoxic cells and a tumor cell. Antibody-drug conjugates (ADCs) also represent an important class of biotherapeutic that have emerged as a promising treatment for cancer. ADCs combine the targeting specificity of antibodies with potent cytotoxins, but unlike bispecific and chimeric proteins, ADCs are obligate conjugates that require the chemical ligation of the drug payload to a protein. The most commonly used conjugation methods exploit the sulfur group of cysteines or the amine group of lysines. Cysteine-based conjugates, however, have shown instability resulting in a premature release of toxin. Likewise, conjugates targeting lysines have been generated using heterobifunctional linkers that contain a succinimide ester and a maleimide. The ester group allows a stable conjugation to the primary amine of lysine residues of the antibody. The maleimide reacts with a sulphydryl of the payload, forming a thioether that is susceptible to uncoupling by reverse-Michael addition. To address this limitation, site-specific conjugates were developed using engineered cysteines encoded at key sites, which have proven successful in reducing the instability and controlling the drug loads on antibodies. The reducing conditions needed for cysteine conjugations can also disrupt disulfide bonds necessary for proper protein structure, and therefore are not amenable for use in all proteins. Thus, methods that allow the introduction of unique chemical moieties, enabling bio-orthogonal conjugation chemistries that are not susceptible to uncoupling and do not require conjugation conditions that alter protein structure, have been developed through the incorporation of non-natural amino acids (nnAAs), glycoengineering and enzymatic modifications of peptide tags.

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The great advantage of nnAAs is the sheer variety of functional chemical moieties, and technologies that are available for site-specific incorporation into proteins. While many have been designed to enable conjugation chemistries (e.g., Diels-Alder, click cycloaddition, and by oxime or hydrazone formation), others have been used to examine protein function, protein–protein interactions, protein folding, or to modify enzymatic activity. For these technologies, the location of the nnAAs is strategically important to enable both easy and efficient functionalization of the protein, but also to preserve its structural integrity. Expression systems in bacterial, yeast, and mammalian cells, as well as in vitro systems, have been developed that offer site-specific incorporation of nnAA with high fidelity. The most popular of these requires the repurposing of a stop codon to encode nnAAs. This is achieved by the heterologous expression of an aminoacyl-tRNA synthetase (aaRS) with specificity for a nnAA, and its cognate tRNA that delivers the nnAA in response to the specified stop codons. This approach enables the site-specific delivery of a nnAA at preselected sites and offers the end user the opportunity to precisely control the site(s) and number of nnAAs.

Several orthogonal aaRS/tRNA pairs have successfully been developed for the incorporation of nnAA in both prokaryotic and eukaryotic expression systems. Methanocaldocroccus jan-naschii TyrRS/tRNA{\textsubscript{tyr}} and yeast PheRS/tRNA{\textsubscript{phe}} were used for the incorporation of nnAAs in E. coli, and E. coli TrpRS/tRNA{\textsubscript{trp}} was evolved for bacterial and mammalian expression. In addition, prokaryotic LeuRS/tRNA{\textsubscript{leu}} and TyrRS/tRNA{\textsubscript{tyr}} are commonly used in mammalian and yeast cells. The Methanosarcina mazei and M. barkeri pylRS/tRNA{\textsubscript{pyl}} are arguably the most versatile pair that supports nnAA incorporation in bacterial, yeast, D. melanogaster, C. elegans and mammalian cells. In each case specific incorporation of a nnAA was observed in response to stop codons. However, expression systems depending on stop codon suppression have historically shown low overall yields. This is due to several factors, including the expression level of the aaRS/tRNA, sequence context of the amber codon, and the function of elongation release factors (RF) that compete with the amber suppressor tRNAs for stop codon recognition resulting in premature termination of protein synthesis and production of a truncated protein. This limitation has been addressed in E. coli systems in various ways: (1) by genetic mutations of RF-1 to eliminate, or reduce, amber codon recognition; (2) by increasing the aaRS/tRNA levels by optimization of promoter elements, codon usage, or the use of multiple repeats of the aaRS/tRNA in expression plasmids; (3) altering the amber codon sequence context to reduce RF-1 recognition; (4) optimization of the tRNA sequence for improved EF-Tu binding and ribosome recognition; through engineering of orthogonal ribosomes specific for the target gene that enable more efficient utilization of amber codons and quadruplet codons; and (6) by expanding the genetic alphabet of cells using unnatural base pairs to assign novel codons to a nnAA.

Mammalian cells are the most common manufacturing platform, offering access to a large variety of protein classes. For the generation of antibodies, mammalian cells are the optimal expression system, as they produce properly folded, functional, and soluble proteins at high yield. Many of the strategies aimed at improving nnAA incorporation listed above are not practical, or simply not possible, in higher eukaryotes. For example, mammalian cells contain a single release factor protein (eRF1) (unlike bacteria which have two) that is essential and recognizes all three stop codons. Thus, stop codon suppression technologies in mammalian cells initially relied on overexpression of the aaRS or tRNA to increase nnAA incorporation efficacy. Studies using transient transfections of mammalian cells determined that high tRNA levels were key to improving nnAA incorporation levels. Further optimizations have aimed to improve the stability and cross-talk of the tRNA with the mammalian translational machinery, improve the introduction of the genetic components into cells, develop promoters to increase tRNA expression, identify permissive sequence context for more efficient amber suppression, as well as overexpress mutant eRF1 with reduced affinity for amber codons. These optimizations have led to significant improvements in the yields of mammalian cells, and more importantly identified key components that affect the efficiency of nnAA incorporation.

Transient transfections of mammalian cells can be used to generate antibodies with nnAAs, but while yields are relatively low, sufficient material can be generated for characterization. In order to reproducibly achieve high productive yields with consistent product quality compatible with manufacturing requirements, stable cell lines are required. One major limitation of mammalian cells in the context of nnAA incorporation is that, unlike bacterial expression systems in which protein production can be completed within a few hours, mammalian expressions require up to 14 days to maximize the production yields. Prolonged amber suppression is thought to be deleterious to cells, resulting in early loss of viability and thus reduced expression times. Nevertheless, high producing platforms capable of g/L yields of nnAA-containing antibodies have been reported. In these cells the orthogonal aaRS and multiple copies of its cognate tRNA were introduced and cells functionally selected. While we have a good understanding of the mechanism behind nnAA incorporation, we do not yet understand the minimal expression requirements of aaRS and its tRNA needed to support the prolonged and high titer expression of stable expressers.

Here we describe the steps used for the generation of a host platform in Chinese hamster ovary (CHO) cells to produce biotherapeutics containing nnAAs, and for the first time, identify the critical components needed for high efficiency of nnAA incorporation. We also show that the process is robust, resulting in the generation of two platform host lines that are each capable of high titer expression of nnAA-containing IgG. Furthermore, we developed a scale-up process that is compatible with established upstream platform perfusion processes, suitable for manufacturing and commercialization.
Results

**Generation of stable platform hosts for the incorporation of non‐natural amino acids**

The incorporation of a nnAA requires engineered cells expressing an orthogonal aaRS/tRNA pair with specificity for the nnAA. In an effort to generate a highly efficient system, we generated stable platform hosts expressing the pyrrolysine tRNA synthetase (pylRS) and its cognate tRNA (tRNApyl) derived from archaeabacterial *Methanosarcina mazei*, an enzyme previously shown to mediate nnAA incorporation in mammalian cells. Stable cell lines armed with this system are a desirable tool for manufacturing because it represents the starting point for the construction of expression cell lines. First, we constructed a vector bearing expression cassettes for PylRS under the control of a CMV promoter, as well as 18 tandem repeats of tRNA under the U6 snRNA promoter. The vector also contained a puromycin selection marker for stable cell line selection. Following transfection into AstraZeneca‐proprietary suspension CHO cells, the transfectants were plated in 96‐well plates and subjected to selection in puromycin‐containing media. Surviving colonies were expanded and screened for amber suppression activity using a high throughput functional assay. This required the transfection of an mRNA reporter probe encoding a red fluorescent protein (RFP)‐green fluorescent protein (GFP) fusion containing an amber codon between the two genes (RFPambGFP) (Figure 1a). Transfected cells were exposed to the nnAA (S‐2‐amino‐6‐((2‐azidoethoxy) carbamoylamino) hexanoic acid (Figure 2a, AzK) to enable amber suppression. With this probe, all transfected cells express RFP, but only cells capable of amber suppression (and nnAA incorporation) also express the GFP reporter (Figure 1b).

Fluorescent protein expression was quantified by flow cytometry in a total of 384 colonies. Two criteria were applied to assess the amber suppression activity. The colonies were ranked based on: 1) the ratio of the geometric means of GFP (amber suppression) to RFP (total expression) (Figure 2b), and 2) the percentage of dual positive cells (Figure 2c). Among the top 50 candidate hosts, 35 platform hosts showed reasonable growth properties and amber suppression activity. These were further screened for stable IgG expression in pool format using an IgG expression plasmid containing an amber codon in the variable domain of the heavy chain (HC) at position 44 (IgGamb), as well as encoding the light chain of the IgG, and the glutamine synthetase (GS) selection marker. With this construct, only cells capable of amber suppression produce a secreted antibody that can be quantified from the growth medium. While most of the hosts were amber suppression positive, based on the mRNA reporter assay, very few showed high titer IgG expression (Figure 2d). Indeed, only pools from three hosts (#27, 38 and 43) demonstrated IgG titers above 100 mg/L, and only one pool from host 43 was capable of producing titers exceeding 1 g/L. Despite the high level of amber suppression activity in this host in medium containing nnAA, no disadvantage in growth was observed in its absence, relative to the parental cell line. These data show that, while the fluorescent reporters are useful tools to identify amber suppressors and reduce the screen size, screening by IgG expression was critical for finding high‐performance amber suppression platform hosts.

**Genetic characterization of nnAA platform hosts**

To learn what factors govern the efficiency of amber suppression, we quantified the gene copy number and mRNA

![Figure 1. mRNA reporter assay for screening amber suppression positive stable isolate hosts. (a) Schematic representation of the process used to generate and identify amber suppression competent cells. CHO cells were first transfected with a plasmid encoding PylRS and tRNApyl and subjected to a selection step by growth in puromycin‐containing medium. Surviving isolates were transfected with mRNA encoding RFPambGFP and cells were exposed to AzK. Cells capable of efficient amber codon suppression (positive) express an RFP‐GFP fusion and are positive for both fluorophores. Cells lacking amber suppression activity (negative) express only RFP. (b) A stable isolate identified as positive for amber suppression activity was transfected with the RFPambGFP mRNA and incubated in the presence or absence of AzK. Cells were imaged by confocal microscopy and flow cytometry. Amber suppression and incorporation of AzK was observed in presence of PylRS and tRNApyl. No GFP expression was observed in absence of AzK.](image-url)
expression of tRNA_{Pyl} and PylRS by quantitative polymerase chain reaction (QPCR) in both high and low IgG-expressing platform hosts (Table 1). Since the pylRS and tRNA_{Pyl} genes are linked, it was not surprising to find a correlation between PylRS and tRNA_{Pyl} gene copy numbers (R-square value of 0.9). Indeed, the pylRS:tRNA_{Pyl} ratios of approximately 1:18,

Table 1. Genetic characterization of amber suppression platform hosts.

| Host ID | Average doubling time (h) | PylRS Copy Number | tRNA_{Pyl} Copy Number | PylRS expression ratio to 18s | tRNA_{Pyl} expression ratio to 18s | % Mature tRNA | % Unprocessed tRNA | Top pool titer (mg/L) |
|---------|---------------------------|-------------------|------------------------|-------------------------------|-------------------------------|----------------|---------------------|-----------------------|
| Parental CHO | 33.3 | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 53 | 25.3 | 2.2 | 42.4 | 0.0045 | 1.54 | 85.6 | N/A | 137 |
| 174 | 39.6 | 0.8 | 14.6 | 0.0036 | 1.12 | 96.1 | 3.9 | 119 |
| 19 | 27.6 | 1.2 | 18.0 | 0.0034 | 0.52 | 95.4 | 4.6 | 147 |
| 27** | 30.0 | 4.6 | 196 | 0.0186 | 6.1 | 97.2 | 2.8 | 540 |
| 30 | 30.1 | 1.2 | 22.3 | 0.0027 | 1.09 | 95.7 | 4.3 | 317 |
| 33 | 24.5 | 0.3 | 7.2 | 0.0023 | 0.63 | 95.6 | 4.4 | 90 |
| 38** | 28.0 | 7.2 | 311 | 0.0335 | 15.5 | 96.3 | 2.7 | 705 |
| 41 | 33.4 | 0.5 | 7.5 | 0.0019 | 0.34 | 96.2 | 3.8 | 360 |
| 43** | 28.4 | 11.5 | 357 | 0.0341 | 9.2 | 93.5 | 6.5 | 1036 |
| 48 | 46.9 | 1.3 | 26 | 0.0056 | 0.42 | 86.8 | 13.2 | 207 |

** Top three hosts based on genetic characteristics and IgG titer
encoded in the input plasmid, were observed in most of the hosts except for hosts 27, 38 and 43, where the tRNA\textsubscript{pyl} gene copy numbers were elevated relative to the PyrRS. Interestingly, mRNA analyses revealed that these hosts also showed elevated tRNA\textsubscript{pyl} and PyrRS expression and the highest IgG\textsubscript{amb} titers, suggesting that higher system expression improves amber suppression potential. In addition, a high correlation (R-square value of 0.9) between gene copy numbers and IgG titer was observed (Figure 3a,b). In an effort to improve the homogeneity of the host populations and to isolate clonal platform hosts with the highest potential for amber suppression, a round of cloning was conducted using ClonePix FL\textsuperscript{77} and derived clones were characterized for amber suppression efficacy as described above. Thus, clones isolated from host 43 were expanded and both tRNA\textsubscript{pyl} and PyrRS copy numbers and IgG\textsubscript{amb} titers were determined (Figure 3c,d). The isolated clones contained about 10 PyrRS genes, between 200 and 300 tRNA\textsubscript{pyl} genes, and supported IgG expression levels of >1.2 g/L. This is in line with previous estimates for highly efficient amber-suppressing cells.\textsuperscript{59} The top clonal host, with an IgG\textsubscript{amb} pool titer of 2.0 g/L, contained the highest copy number for tRNA\textsubscript{pyl} (>600 copies) and PyrRS (>25 copies). The correlation between the tRNA\textsubscript{pyl} and PyrRS copy number and IgG titer among the clonal hosts was reduced (R-squared of 0.68), possibly due to the less diverse nature of the parental host 43 contributing to the cluster of clonal hosts with similar tRNA\textsubscript{pyl} and PyrRS copy numbers, as well as IgG productivity. These data clearly show that elevated PyrRS/tRNA\textsubscript{pyl} gene copy numbers and expression levels support higher amber suppression activity and point to PyrRS and tRNA\textsubscript{pyl} copy number analyses as a key metric for characterizing platform hosts.

**Characterization of IgG expressing cell lines generated in nnAA host platforms**

Having identified platform hosts capable of amber suppression and high titer expression of IgG\textsubscript{amb}, we set out to quantify the efficiency of nnAA incorporation in stable IgG\textsubscript{amb} cell lines generated from these hosts. To do this, we stably expressed IgG in two different hosts containing high (host 43) and low (host 27) copy numbers for tRNA\textsubscript{pyl} and PyrRS. The IgG expression construct contained an amber codon at heavy chain position K274 that generates a truncated IgG in the absence of amber suppression, but upon readthrough of the amber codon, a full-length IgG is produced. Because the antibody used contains a Fab-HC belonging to the VH3 family, capable of protein A binding, the full-length IgG (detected using Fc-specific antibodies) and total IgG (detected using protein A sensors) could be quantified by biolayer interferometry with separate assays. Thus, the ratio of full-length/total IgG reflects the efficiency of nnAA incorporation by the system. Cell performance of the primary isolates were assessed by monitoring viable cell density (VCD) (Figure 4a), viability (Figure 4b), and IgG titers for both full-length and truncate forms over a 14-day fed-batch expression using AstraZeneca-proprietary media and nutrition-enriched feed (Figure 4c). Amber suppression was induced by the addition of 2 mM AzK to the medium on day 4 of the fed-batch culture.

We observed that IgG-expressing cell lines derived from host 27 and 43 maintained high VCDs and cell viability during the fed-batch process (Figure 4a,b). All expresser cell lines tested produced equivalent levels of total IgG (1.3–1.6 g/L). The cell

![Figure 3. Correlation between PyrRS and tRNA\textsubscript{pyl} copy numbers and IgG Titer.](image-url)
line derived from host 27 (27–274#2), containing only 89 copies of tRNA\textsubscript{pyl} and 3 copies of PylRS (Table 2), produced 134 mg/L of full-length IgG, representing an amber suppression efficiency of 10% (Figure 4c). Western blot analyses of crude supernatants show that the majority of the product is truncated IgG, whereas the full-length IgG is almost undetectable (Figure 4d). Interestingly, cell line 43–274#4 derived from host 43, containing 280 copies of tRNA\textsubscript{pyl} and 10 copies of PylRS (Table 2), showed 42% amber suppression efficiency and produced much higher levels (613 mg/L) of full-length IgG (Figure 4c) that is clearly detected by Western blotting (Figure 4d). The third cell line 43–274#5, containing 161 copies of tRNA\textsubscript{pyl} and 6 copies of PylRS (Table 2), showed 20% amber suppression activity and produced 344 mg/L full-length IgG (Figure 4c). The genetic characteristics of the cell lines, full-length and truncated IgG expression levels, and their corresponding amber suppression activity are summarized in Table 2. Interestingly, the differences in amber codon suppression efficiency were not related to lack of tRNA\textsubscript{pyl} maturation, as all cell lines demonstrated equivalent levels (~85%) of mature (processed) tRNA\textsubscript{pyl} (Table 3). However, it is clear from these data that the copy number and expression levels of tRNA\textsubscript{pyl} and PylRS determine amber suppression efficiency.

**Expression ratios are expressed relative to \(\beta 2M\) expression.

### Table 2. pylRS and tRNA expression analysis and amber suppression efficacy of top three platform hosts.

| Cell Line ID | tRNA\textsubscript{pyl} Copy Number | PylRS Copy Number | Total IgG Expression (mg/L) | Full Length IgG Expression (mg/L) | Amber suppression efficiency |
|--------------|-------------------------------|------------------|-----------------------------|---------------------------------|-----------------------------|
| 27–274#2    | 89.6                          | 3.1              | 1322                        | 134                             | 10.1%                       |
| 43–274#4    | 280.2                         | 10.6             | 1436                        | 613                             | 42.6%                       |
| 43–274#5    | 161.2                         | 6.0              | 1668                        | 344                             | 20.6%                       |

**Expression ratios are reported relative to 18S expression.

### Table 3. tRNA and pylRS expression analysis of IgG (K274 amber) stable expressers.

| Cell Line ID | Processed tRNA\textsubscript{pyl} Expression Ratio | Unprocessed tRNA\textsubscript{pyl} Expression Ratio | Mature to Unprocessed tRNA Ratio (%) | PylRS Expression Ratio |
|--------------|-----------------------------------------------|----------------------------------|-----------------------------------|------------------------|
| 27–274#2    | 0.0296                                         | 0.0035                           | 88%:12%                          | 0.00004                |
| 43–274#4    | 0.0956                                         | 0.0150                           | 84%:16%                          | 0.00006                |
| 43–274#5    | 0.0296                                         | 0.0044                           | 85%:15%                          | 0.00003                |

**Host platform generation with mutated PylRS and tRNA\textsubscript{pyl} for expansion of repertoire of nnAAs incorporation in protein sequences**

To expand the repertoire of nnAAs that can be introduced into proteins, we applied the same screening strategy to generate hosts capable of introducing nnAAs containing cyclopentadiene moieties such as CpHK and SCpHK (Figure 5a). These lysine derivatives enable Diels-Alder cycloaddition, a rapid, metal free, and efficient reaction useful for the conjugation to dienophile bearing payloads (e.g., maleimide). The incorporation of CpHK and SCpHK, however, requires a PylRS containing a mutation (Y306A) that
expands the amino acid binding pocket, enabling the capture of CpHK and SCpHK. Thus, a plasmid encoding pylRSY306A (Y306A) and 18 tRNApyl repeats was constructed and stably transfected into AstraZeneca proprietary suspension CHO cells.\textsuperscript{73,74} Transfected cells were selected and characterized as previously described. Figure 5b shows the titer distribution of stably expressed IgGamb in stable pool format. Four platform hosts produced more than 500 mg/L IgG (IgGamb containing an amber codon at position HC44 was used) from stable pools (Figure 5c). The comparison of the platform host generation process expressing the wildtype (WT) and Y306A is summarized in Table 4. The screening profiles for the generation of highly active amber suppression-enabled host platforms show the reproducibility of the screening strategy. It also suggests that finding hosts with high amber suppression activity is a rare event and requires high throughput functional screening. The genotypic and phenotypic characteristics of the four top Y306A hosts are summarized in Table 5. Based on the IgG productivity, PylRS and tRNApyl copy numbers, and population doubling time, host Y306A36 was chosen for further development as a manufacturing host. Interestingly, the Y306A hosts were shown to incorporate both CpHK and SCpHK as well as AzK, albeit with lower efficiency, making these hosts useful for the characterization of different conjugation chemistries.

### Stability of nnAA platform hosts for amber suppression activity and IgG expression

The stability of an expression host is an important consideration for the selection of a manufacturing platform to ensure consistent production levels and product quality. To evaluate this the top platform hosts for each of our campaigns, hosts 27, 38 and 43, the top three clonal hosts 43#3, 43#8 and 43#37, and Y306A36 were routinely passaged and cells were cryo-preserved after 10 (E, early) and 60 (L, late) population doublings (PDLs). Each of the cryopreserved platforms were revived and stably transfected with an IgGamb expression construct encoding IgG containing an amber codon at position 44 of the HC. Twelve IgGamb expressing stable pools were generated and evaluated for productivity (Table 6). We

| Table 4. Comparison of host identification metrics from two independent cell line engineering campaigns. |
|-------------------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Host platform | Total colonies screened | Positives post functional screen | Screened by IgG expression | Number of potential good hosts | Positive hits (%) |
|----------------|-----------------|-----------------|-----------------|--------------------|-----------------|
| WT | 384 | 50 | 35 | 3 | 0.78 |
| Y306A | 216 | 64 | 38 | 5 | 2.3 |

| Table 5. pylRS and tRNA expression analysis for top pylRSY306A hosts. |
|-------------------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Host ID | Average doubling time (h) | PylRS Copy Number | tRNApyl Copy Number | PylRS expression ratio to 18s | tRNApyl expression ratio to 18s | % Mature tRNA | % Unprocessed tRNA | Top pool titer (mg/L) |
|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Y306A22 | 39.3 | 5.3 | 210.0 | 0.0018 | 0.94 | 94.4 | 5.6 | 592 |
| Y306A36 | 38.1 | 10.3 | 343.0 | 0.0014 | 0.97 | 86.2 | 13.8 | 696 |
| Y306A37 | 42.1 | 30.9 | 1103.2 | 0.0071 | 2.73 | 92.7 | 7.3 | 687 |
| Y306A49 | 45.9 | 12.7 | 469.6 | 0.0043 | 1.92 | 92.8 | 7.2 | 773 |
observed a decrease in the productivity of cells with increased PDL. The platform Y306A36 was an exception, retaining its productivity even after 60 PDLs. To better understand the mechanism behind this drop in productivity, we assayed each platform for tRNAPyl and PylRS copy number. WT PylRS hosts 27 and 38 lost PylRS and tRNAPyl copies over 60 generations and were not evaluated further for IgG expression (Table 6). The host 43 showed a 27% drop in tRNAPyl and PylRS genes copy numbers in late passage cells, which contributed to a nearly 40% drop in average titer in stable pools. Whereas 43#8 showed stability in the PylRS and tRNAPyl copy numbers, the mean IgG titer dropped almost 50% in late, compared to early passage cells, even though the specific productivity (QP) remained consistent (Supplemental Figure 1d). The drop in overall titer was associated with reduced growth rate and early cell death of the late passage cells in a 14-day fed-batch expression (Supplemental Figure 1a and b). The Y306A36 host retained its PylRS and tRNAPyl copy numbers and IgG productivity in stable pools. The drop in tRNAPyl and PylRS mRNA expression and increased levels of unprocessed tRNAPyl in late passage cells of Y306A36 host did not affect the median titers of the stable IgG pools.

**Process development strategies for IgG production using nnAA hosts in perfusion bioreactors**

The use of orthogonal aaRS/tRNA for the incorporation of nnAAs has proven to be a very precise method for delivering a nnAA at preselected sites. However, the major limitation of nnAA technologies has been the low productivity in cell-based systems. Previous reports demonstrating >1g/L yields show that the limitations of this system can be overcome in stable cells and with careful cell line selection. To further improve the robustness of this system, and further increase the expected overall titers, we developed a process for the expression of nnAA-containing proteins using perfusion bioreactors. Perfusion systems allow for the addition of fresh media and nutrients and the simultaneous removal of cell waste to maintain optimal growth and expression conditions. Thus, stable cells expressing an antibody directed against a human epidermal growth factor receptor 2 (HER2)/neu, containing an amber codon encoded in the antibody constant heavy domain 2 (CH2), were generated in host 43 and Y306A36 hosts and IgG-expressing stable primary isolates were subjected to perfusion fermentations. The site of incorporation can affect expression levels of the system; in this assessment, we selected a CH2 domain site that represents a challenging position for incorporation efficiency and is functionally significant for ADC assembly. The expression line derived from host 43 was exposed to AzK (V/AzK), CpHK (V/CpHK) or SCpHK (V/SCpHK) starting on day 5 of the fermentation.

In each culture, the antibody productivity, amber suppression efficiency, cell viability, and viable cell density (VCD) were monitored over a 24-day period (Figure 6a–d). We observed that S/AzK achieved very high cell densities (1 x 10^8 cells/mL), whereas the V/AzK, V/CpHK and V/SCpHK maintained a VCD of 5 x 10^7 cells/mL (Figure 6a). The high VCD of S/AzK cell line is most likely related to the characteristics of the platform host and independent of the nnAA incorporation system. Nevertheless, perfusion fermentation allows the generation of a higher biomass than is typically achieved by conventional methods (1–10 x 10^6 cells/mL). Despite the high cell densities, the viability of all the cultures was maintained above 80% for 10 days with a gradual decline over the remaining period. It should be noted that V/SCpHK cells showed an earlier decline than other cultures (Figure 6b). This more rapid decline however, was not related to the efficacy of amber suppression (calculated as $\frac{\text{Full length IgG}}{\text{Total IgG (Truncated + Full length)}}$), as V/CpHK showed a higher efficiency of nnAA incorporation than other cell lines. Although, the amber suppression efficiency was higher in V/CpHK (40%) compared to V/SCpHK (23%) (Figure 6d), the full-length IgG normalized productivity of both cell lines was 3.0 g/L. Due to the lower efficiency of AzK incorporation by the Y306A36 host, V/AzK showed a lower efficiency (20%) than other cells and resulted in a normalized titer of 1.5 g/L (Figure 6c). AzK incorporation in S/AzK was also around 40% efficient (Figure 6d) and produced about 2.5 g/L of full-length IgG (Figure 6c).

**Table 6.** Stability of pylRS and tRNAPyl expression in early and late passage populations.

| Sample | tRNAPyl Copy number | PylRS Copy number | tRNAPyl expression ratio | PylRS expression ratio | Matured tRNAPyl | Unprocessed tRNAPyl | Average pool titer (mg/L) |
|--------|---------------------|--------------------|--------------------------|------------------------|-----------------|---------------------|--------------------------|
| 27L    | 196                 | 4.6                | 1.50                     | 0.0046                 | 96.9%           | 3.1%                | N/A                      |
| 27E    | 69                  | 2.2                | 0.02                     | 0.0001                 | 72.1%           | 27.9%               | N/A                      |
| 38L    | 311                 | 7.2                | 1.36                     | 0.0048                 | 95.6%           | 4.4%                | N/A                      |
| 38E    | 71                  | 2.3                | 0.30                     | 0.00004                | 83.7%           | 16.3%               | N/A                      |
| 43L    | 259                 | 8.8                | 1.40                     | 0.0014                 | 95.7%           | 4.3%                | 878.8 ± 214.0            |
| 43E    | 194                 | 6.5                | 1.43                     | 0.0019                 | 96.1%           | 3.9%                | 528.2 ± 153.4            |
| 43L3E  | 239                 | 9.8                | 1.46                     | 0.0013                 | 88.3%           | 11.7%               | 742.2 ± 236.5            |
| 43L3L  | 166                 | 6.2                | 0.65                     | 0.0006                 | 89.5%           | 10.5%               | 379.5 ± 60.3             |
| 43L8E  | 224                 | 9.1                | 1.22                     | 0.0010                 | 92.2%           | 7.8%                | 1144.6 ± 271.9           |
| 43L8L  | 285                 | 12.1               | 1.22                     | 0.0005                 | 95.9%           | 4.1%                | 542.4 ± 82.2             |
| 43L37E | 445                 | 16.4               | 0.96                     | 0.0007                 | 91.0%           | 9.0%                | 799.5 ± 203.8            |
| 43L37L | 126                 | 4.7                | 0.25                     | 0.0004                 | 93.3%           | 6.7%                | 138.9 ± 36.42            |
| Y306A  | 377                 | 12.8               | 1.20                     | 0.0016                 | 90.1%           | 9.9%                | 315.0 ± 84.0             |
| Y306A  | 354                 | 14.8               | 0.64                     | 0.0008                 | 85.9%           | 14.1%               | 462.1 ± 127.5            |

Figure 6a–d. This more rapid decline however, was not related to the efficacy of amber suppression (calculated as $\frac{\text{Full length IgG}}{\text{Total IgG (Truncated + Full length)}}$), as V/CpHK showed a higher efficiency of nnAA incorporation than other cell lines. Although, the amber suppression efficiency was higher in V/CpHK (40%) compared to V/SCpHK (23%) (Figure 6d), the full-length IgG normalized productivity of both cell lines was 3.0 g/L. Due to the lower efficiency of AzK incorporation by the Y306A36 host, V/AzK showed a lower efficiency (20%) than other cells and resulted in a normalized titer of 1.5 g/L (Figure 6c). AzK incorporation in S/AzK was also around 40% efficient (Figure 6d) and produced about 2.5 g/L of full-length IgG (Figure 6c).
IgG produced by the perfusion process shows efficient conjugate formation and potency in vitro

To demonstrate that the products of the perfusion expressions efficiently generated antibodies with site-specific nnAA incorporation, the IgG products were subjected to a conjugation reaction with the toxin tubulysin. To do this, each perfusion culture was collected, and the antibodies purified by affinity chromatography. The purified antibodies were then combined with tubulysin payloads armed with linker groups that allow specific conjugation chemistries for the integrated nnAAs, maleimide (for conjugation to CpHK and SCpHK) or dibenzylcyclooctyne (DBCO) (for conjugation to AzK). Due to the increased mass of antibody-drug adducts, HC conjugates show a retardation in gel mobility that is readily observed by SDS-PAGE and Coomassie staining. We observed efficient conjugate formation in all cases, suggesting efficient nnAA incorporation (Figure 7a). Furthermore, the deconvoluted masses of reduced antibodies, and their corresponding ADCs, measured by mass spectrometry, show mass differences that are consistent with the addition of the corresponding payloads (difference of 1074.6 Da for monoclonal antibodies (mAbs) with AzK and 1091.6 Da for mAbs with CpHK or SCpHK). The data also show that the reactions are complete, and that each HC was modified by a single warhead (Figure 7b). The site-specific nnAA incorporations and conjugations were confirmed by peptide mapping. Nonspecific conjugation was not observed (Supplement Figure 2).

The glycoforms for naked antibodies and their corresponding ADCs from peptide mapping data are summarized in Table 7. Interestingly, the major glycoforms are fucosylated biantennary structures (G0f, G1f, G2f) without sialic acid for the host 43-derived expresser and with sialic acid (G1f+NAc, G2f+NAc, G2f+2NAc) for the Y306A-derived cell line. However, there is no difference in glycan profiles before and after conjugation for all samples. Further characterization of IgG products derived from other Y306A platform hosts suggests that the excess sialylation was an independent characteristic of host Y306A36 platform and unassociated with the introduction of the PylRS gene.

The constructed ADCs were tested to assess their potency and specificity in vitro. For these assays, HER2-positive SK-BR-3 and HER2-negative MDA-MB-468 cell lines were cultured in vitro with the indicated ADCs and controls, and their viability assessed after 3 days (Figure 7c). The data show that all the anti-HER2 ADCs are cytotoxic to high Her2-expressing cells, SK-BR-3 (IC50 = 4–7ng/ml), but greatly reduced toxicity to HER2-negative cells, MDA-MB-468 (IC50 >3600ng/ml) (Table 8).

Table 8. In vitro potency (IC50 ng/mL) of Tubulysin conjugates on Her2-positive SK-Br-3 cells.

| IgG sample | Payload (Tubulysin) | IC50 (ng/ml) |
|------------|---------------------|--------------|
| HER2 S/AzK | +                   | 4.08         |
| HER2 V/AzK | +                   | 7.25         |
| HER2 V/CpK | +                   | 5.71         |
| HER2 V/SCpK| -                   | 429          |
| HER2 V/SCpK| +                   | 4.82         |
| R347/AzK  | +                   | >3600        |
Figure 7. Functional assessment of nnAA bearing antibodies. Antibodies purified from each of the perfusion cultures were purified and characterized for conjugation efficacy and ADC function. (A) Affinity-purified antibodies were conjugated to tubulysin warheads containing alkyne- (DBCO; for AzK IgGs) or maleimide- (for SCpHK and CpHK) bearing linkers. Unconjugated (-) and conjugated samples were resolved by SDS-PAGE and stained with Coomassie. A retardation in gel mobility is observed in the HC, but not the LC, of conjugated samples, suggesting near-complete conjugate formation. (B) Reduced antibodies and ADCs were analyzed by mass spectrometry. ADCs show an intact HC mass increase corresponding to the mass of the DBCO-tubulysin (1074.6 Da) or maleimide-tubulysin (1091.61 Da) adducts indicating the addition of a single payload to the HC. No unreacted material was observed in ADC samples. C. and D. The constructed ADCs were functionally tested in an in vitro cytotoxicity assay. Antibodies and ADCs were incubated with HER2 positive (Sk-Br-3) (c) and negative (MDA-MB-468) (d) cell lines and their viabilities were assessed. V/CpHK (-) in C represents HER2 mAb without a toxic payload. All ADCs show potent and specific on target killing.
Moreover, a non-HER2 targeting control antibody (R347) expressed to contain AzK and conjugated with tubulysin demonstrated low cytotoxicity to SK-BR-3 cells (IC50 > 3600). These data collectively demonstrate the high potency and specificity of cytotoxicity of the conjugated mAbs derived from both WT and Y306A platform hosts.

Intracellular localization and detection of PylRS in secreted mAb and purified IgG samples

The overexpression of PylRS in manufacturing host cell lines raises an immunogenicity risk if this archaeobacterial-derived protein contributes to the host cell protein load in a mAb bioprocess or contaminates the final product. To begin to address this possibility, we first examined the subcellular distribution of PylRS by immunofluorescence confocal microscopy (Figure 8a). Antibody-expressing cells were fixed and stained for PylRS, a plasma membrane protein (Atp1a1) and nuclei (DAPI). We observed that PylRS co-localizes with the plasma membrane marker, but also shows cytoplasmic staining (Figure 8a). To assess whether PylRS is secreted and whether it co-purifies with IgG, we examined cell lysates (50 µg), cell supernatants (1 µg) and protein A purified IgG (1 µg) by Western blot analysis. PylRS was detected in cell supernatants of fed-batch cultures harvested on day 6, indicating that some of the expressed PylRS was escaping the cells. It is unclear whether the PylRS is secreted by the cells or is present in the supernatant as a result of cell lysis. It is notable that the cultures showed high viability at the time of sampling (98% viable), suggesting that the extracellular PylRS may not be entirely due to cell lysis. Nevertheless, PylRS was not detected in protein A-purified IgG (Figure 8b). These findings suggest that the potential for contamination of purified IgG product with PylRS protein is minimal, limiting the risk of immunogenicity.

Table 7. Glycosylation profiles of antibodies generated by mAA host cells.

| Glycoforms | S/AzK | S/AzK+ Tubulysin | V/AzK | V/AzK+ Tubulysin | V/CpK | V/CpK+ Tubulysin | V/SCpK | V/CpK+ Tubulysin |
|-----------|-------|-----------------|-------|-----------------|-------|-----------------|-------|-----------------|
| G0-GN     | 2.9   | 2.8             | 1.5   | 1.1             | 1.4   | 1.5             | 2.0   | 2.2             |
| G0f-GN    | 3.6   | 3.6             | 3.1   | 4.9             | 2.6   | 2.7             | 3.4   | 3.7             |
| G1-GN     | 1.2   | 1.2             | 0.4   | 0.5             | 0.3   | 0.3             | 0.4   | 0.4             |
| G1f-GN    | 1.9   | 2.0             | 2.6   | 6.1             | 1.3   | 1.5             | 1.7   | 1.8             |
| G1f-GN+NAc| 0.5   | 0.4             | 2.1   | 2.7             | 1.0   | 0.8             | 1.3   | 1.0             |
| G0       | 8.1   | 8.1             | 0.4   | 0.4             | 0.3   | 0.2             | 0.4   | 0.3             |
| G0f      | 40.8  | 40.8            | 6.2   | 5.1             | 5.7   | 5.1             | 5.1   | 6.0             |
| G1       | 2.9   | 2.9             | 0.6   | 0.6             | 0.5   | 0.5             | 0.6   | 0.7             |
| G1f      | 22.8  | 22.8            | 16.6  | 16.1            | 17.1  | 18.9            | 18.3  | 20.0            |
| G2f      | 3.8   | 3.8             | 13.6  | 15.4            | 14.5  | 16.3            | 14.0  | 14.8            |
| G1f+NAc  | 0.1   | 0.1             | 7.1   | 5.8             | 9.3   | 9.4             | 8.8   | 8.8             |
| G2f+NAc  | 0.3   | 0.3             | 17.2  | 16.6            | 19.6  | 19.9            | 16.8  | 16.7            |
| G2f+2NAc | 0.1   | 0.2             | 16.3  | 13.9            | 16.2  | 16.6            | 13.7  | 13.7            |
| M3       | 1.5   | 1.4             | 0.7   | 1.3             | 0.1   | 0.1             | 0.2   | 0.2             |
| M5       | 10.1  | 9.9             | 10.2  | 11.6            | 8.0   | 8.1             | 10.9  | 11.3            |
| M6       | 0.9   | 1.0             | 0.7   | 0.8             | 0.9   | 1.0             | 1.1   | 1.2             |
| M7       | 0.4   | 0.4             | 0.2   | 0.2             | 0.4   | 0.4             | 0.4   | 0.4             |
| M8       | 0.2   | 0.1             | 0.2   | 0.2             | 0.3   | 0.3             | 0.4   | 0.4             |
| aglycosylation | 3.3 | 4.0 | 1.0 | 1.1 | 1.2 | 1.2 | 1.0 | 1.1 |

**S/AzK was generated in host 43 expressing WT PylRS whereas N/AzK/CpK/CpK were generated in Y306A36 host expressing mutant PylRS

Figure 8. Intracellular localization of PylRS. (a) Localization of recombinant PylRS by confocal microscopy in primary isolates of IgG expressors in host 43 platform hosts. Cells were fixed and stained with anti-FLAG antibodies (PylRS) and the membrane protein Atp1p. DAPI was used to stain the nuclei. PylRS seems to localize to the cytoplasm of cells. (b) To examine whether PylRS is secreted, 50 µg of whole-cell lysates (L), 1 µg crude supernatants (S), and 1 ug of purified IgG derived from host 43 expressers were resolved by SDS-PAGE and examined by Western blotting. FLAG-tagged PylRS (upper panel) was detected using anti-FLAG antibody. IgG HC and LC were examined as loading controls (lower panel).
Methods for genetic code expansion are valuable strategies for introducing novel functionality into proteins. The most common way of introducing nnAAs is through stop codon suppression methods that require the function of orthogonal aaRS/tRNA pairs with specificity for a nnAA. This method has been shown to reliably deliver nnAAs to specific sites of a target protein. A variety of nnAAs with different functional moieties have been described that are useful for protein engineering, including altering protein structure and immunogenicity, stabilizing protein interactions, and mimicking post-translational modifications, but their most common application is to enable biorthogonal conjugations. In particular, nnAA-based conjugates have been shown to have superior stability as ADCs, where the loss of payload in circulation adversely impacts both the safety and efficacy of drugs. While these provide a significant improvement over conventional ADCs, low productivity of expression systems has limited their development. Indeed, few examples of high titer antibody expression are available.

We developed a two-step screening strategy to identify a high-performance nnAA host platform. Cells were initially screened in a high throughput fashion with an amber suppression-dependent dual fluorescent mRNA reporter followed by screening for high titer IgGamb expression (Figure 2c). The fluorescent mRNA reporter was effective at identifying amber suppression competent cells (Figures 1a,b and Figure 2a,b), but, due to the limited dynamic range of fluorescence levels, it did not effectively discriminate cells with very high amber suppression activity. Thus, a secondary screen was implemented using an amber containing IgG to identify cells that were capable of high titer IgG expression.

Once the platform hosts capable of generating high titer, amber codon dependent expression were successfully identified, we characterized the hosts for the content of pylRS and tRNApyl using QPCR-based methods, we determined a correlation between IgGamb productivity and tRNApyl and pylRS copy number (Table 1). Interestingly, in these cells gene copy number is a better indicator for IgGamb yield than tRNApyl and pylRS mRNA expression levels. Indeed, loss in IgG productivity due to phenotypic instability of the platform hosts can be correlated with reduced tRNApyl and pylRS gene copy numbers (Table 4). This is in contrast to conventional mAbs where mRNA levels correlate with titers. The trend may not apply to pylRS and tRNApyl levels because they function upstream of the gene of interest, creating a functional separation that provides an opportunity for regulation that we have yet to explain. Nevertheless, the copy number analysis is an important analytical metric to identify hosts with high amber suppression activity.

To establish the reproducibility of the host generation strategy, a second host cell line was developed using an engineered PylRS (Y306A) mutant capable of incorporating an expanded list of nnAAs. Interestingly, the PylRS bearing the Y306A mutation enables the incorporation of AzK, albeit with reduced efficiency, but also incorporates lysine analogues bearing cyclo- and spiro-cyclopentadiene moieties (CpHK and ScpHK). These latter nnAAs enable conjugation reactions in metal-free conditions with dienophiles (e.g., maleimide bearing molecules), allowing a rapid and effective formation of a stable linkage for next-generation ADCs.

The manufacturability of this system was demonstrated by a reproducible, scalable manufacturing process based on perfusion technology that is suitable for both the WT and Y306A platform host-derived IgG-expressing cell lines for the incorporation of AzK, CpHK, and ScpHK nnAAs. These cells were shown to deliver a normalized titer ranging from 1.5 to 3.0 g/L, a reproducible yield that is acceptable for manufacturing. Interestingly, we observed that the cultures tolerated 40% amber suppression efficiency for 20–25 days (Figure 6a-d). A highly efficient amber suppression system in cell lines is necessary to support the expression demands required for the manufacturing of nnAA-containing therapeutic proteins. It should be noted that 25% of the CHO genome encodes proteins terminating with an amber stop codon (Baycin Hizal, unpublished data). While the amber codon is the least used of the stop codons in mammalian cells, it is expected that each of these codons will be suppressed in our host platform upon exposure to nnAA. We expect that global suppression of amber codons will result in the extension of essential cellular proteins that may alter their function and be deleterious to the cells, causing premature cell death and low IgG expression. Global amber codon suppression is mitigated in part by the strict orthogonality of the PylRS/tRNApyl pair and its specificity for nnAAs, which ensures that amber suppression occurs only in the presence of nnAA. If cytotoxicity did occur after the addition of nnAA due to amber suppression of cellular genes, the effects were not immediately observed. Rather, they were delayed 4–7 days, and even then, the loss of viability was relatively slow. The most affected culture was the one exposed to ScpHK that showed an early onset of loss of viability. This, however, is not likely due to amber suppression efficiency, as the ScpHK cells were no more efficient than the CpHK counterpart. The observe loss in viability may be due to cytotoxicity from the nnAA itself and requires additional characterization.

The platform cell lines described here have shown the ability to generate expression cell lines capable of high titer antibody expression in line with manufacturing requirements. However, the stability of the host platform is of critical importance to ensure consistent yields and product quality. In an effort to assess the general stability of the host cells, and the nnAA incorporation system, we opted to age a set of our platform hosts to 60 PDL prior to transfection and isolation of expression pools. Reduced nnAA IgG expression titers observed in platform hosts could be attributed to decreased cell viability and growth (host 43#8) or genetic instability of pylRS and tRNA genes (hosts 43, host 43#3, and host 43#37). In contrast, host Y306A36 retained pylRS/tRNA copy numbers and productivity over this passage number. These data show that genetically stable hosts can be isolated despite the high number and repetitive nature of the gene constructs. However, to consider this as an expression system qualified for manufacturing, additional data on the stability of production cell lines as well as their product quality are needed.

The site of conjugation is an important consideration in the assembly of an ADC because it can affect the conjugation efficiency and potency of the drug. With the amber suppression technology, the site of the nnAA incorporation can also affect the expression yields. The materials generated by the perfusion process here were not selected for their expression potential.
but rather for the functionality of the site. Indeed, we have observed productivity levels of 1.7 and 2 g/L in conventional fed-batch cultures with antibodies bearing nnAAs at other sites. The sites selected for this study showed efficient conjugate formation with tubulysin containing either strain-promoted azide–alkyne cycloaddition (SPAAC) or maleimide chemistry (Figure 7a,b). Additionally, the conjugated material is highly potent and specific to target cells (Figure 7c). We also clearly demonstrated that, even though the archaebacterial PylRS is detected in the cell supernatants, it is not co-purified with secreted IgG products (Figure 8b). Co-expression of the bacterial PylRS in CHO cell lines for therapeutic mAb production is unlikely to pose a threat to patients by provoking immunogenicity to the PylRS protein.

Overall, these new cell lines provide a means for the production of ADCs with novel chemistries for conjugation and open opportunities for exploration of alternative sites of conjugation, not accessible to conventional chemistries, that may address current limitations observed with conventionally constructed ADCs. We show here that stable cell lines capable of genetically encoding nnAAs with high yields require high numbers and expression levels of the suppressor tRNA_pyl and its corresponding pylRS. Indeed, our best platforms demonstrate tRNA_pyl levels that are comparable in expression to 18S rRNA, a highly abundant message found in cells. The levels of pylRS are not in this vicinity, and may not be necessary, but a minimal level of expression is needed to effectively aminoaclylate the available tRNA_pyl. Excess pylRS expression, however, has been shown to reduce nnAA incorporation efficiency. Thus, a balance must be achieved that works cooperatively to potentiate amber suppression at high efficiency without creating unnecessary demands that affect viability and growth. Here we have relied on phenotypic selections to identify suitable host cells. However, further improvements to the efficacy of this expression system are necessary to increase the economy of producing nnAA-containing proteins, but also to enable the incorporation of multiple nnAAs, within a protein, for the construction of ADCs with high drug-to-antibody ratios. This may be achieved by implementing optimizations, such as the use of stabilized tRNA_pyl sequences, overexpressing eRF1 mutations, and selecting optimal codon contexts for amber codons that have been previously described in transient expression systems. These cell lines are not limited to the expression of antibodies and the generation of ADCs, but give access to any target protein that can be expressed in CHO cells. The functionality conveyed by the PylRS and tRNA_pyl pair may also enable the construction of a novel, enhanced therapeutics for more effective treatment of human diseases.

Materials and methods

Transfection of CHO cells using plasmid DNA and synthetic mRNA and high throughput screening for amber suppression activity

Transfections were carried out with an Amaxa Nucleofector II device following the manufacturer’s recommendations and using the Solution V kit (Lonza, Cat# VCA1003). For plasmid DNA transfections, 5 × 10^6 cells were transfected with 5.0 µg DNA prepared using an Endo-free plasmid Maxi kit (Qiagen, Cat# 12362). Synthetic mRNA encoding RFpamb-GFP (TriLink Biotechnologies) transfections were optimized using 1 × 10^6 cells and 1.7–3.4 pmol mRNA construct using program EH118 in a 96-well shuttle (Lonza). However, during high throughput screening, approximately 0.25 × 10^6 cells were transfected using 2.0 pmol synthetic mRNA to obtain optimal transfection efficiency. Nucleofected cells were recovered in 96-well plates containing CD-CHO medium (ThermoFisher Scientific, Cat# 10743029) supplemented with 6 mM L-glutamine (ThermoFisher Scientific Cat# 25030081). 2.0 mM AzK was used for inducing amber suppression activity. Functional screening of amber suppression activity by flow cytometry for detection of dual fluorescent (GFP- and RFp-positive cells) protein expression was carried out at 24-h post nucleofection using an LSRII cytometer (BD Biosciences) and data analyzed using FlowJo software (FLOWJO, LLC).

Generation of IgG-expressing stable pools for the screening of amber suppression positive hosts

CHO cells, derived from CHO K1, adapted to grow in suspension were transfected using Amaza 96-well shuttle (Lonza, Walkersville). For the generation of platform hosts, cells were transfected with a linearized plasmid encoding pylRS, 18 tandem repeats of the tRNA_pyl and a puromycin resistance cassette. Transfected cells were resuspended in CD-CHO medium containing 6.5 µg/ml puromycin, and cells plated into 96-well plates and incubated in a controlled environment incubator at 37°C with 6% CO2. Wells showing colonies were harvested and expanded. Each colony was assessed for amber suppression activity using the RFpamb-GFP construct. Colonies with the best GFP:RFP ratios were assessed for IgG expression. To do this, each colony was transfected with a linear mAb expression plasmid containing an amber codon at position 44 in HC and selected as mini pools in 24-well plates in CD-CHO medium supplemented with Puromycin (ThermoFisher Scientific, Cat# A1113802) and 50 µM methionine sulfoximine (MSX, Sigma-Aldrich, Cat# M5379). Surviving pools were expanded in shaking flasks. Amber suppression was induced in drug-selected mini-pools on day 4 of a 14-day fed-batch assay and IgG expression was monitored in 24 deep-well plates by biolayer interferometry using protein A-coated sensors. AstraZeneca-proprietary medium and feed were used for the fed-suspension assay. Stable hosts were ranked based on IgG expression. Clonal hosts were generated by growing a host pool on semi-solid medium and colonies isolated using ClonePix FL. For the generation of stable IgG expresser colonies, platform hosts were nucleofected with a linearized plasmid encoding the IgG (containing an amber codon at the desired position), and a glutamine synthetase gene. Transfected cells were plated into 96-well plates and grown in CD-CHO medium lacking glutamine and containing 50 µM MSX. Wells showing cell growth were selected and assessed for IgG expression in suspension growth conditions.

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cells were DNase treated using DNA-free kit (Ambion, Cat# AM1906). Genomic DNA (gDNA) was also isolated from a maximum of $5 \times 10^6$ cells kept as frozen pellets using the AllPrep DNA/RNA Mini Kit (Qiagen, Cat# 353445). Total RNA (tRNA, mRNA, and rRNA) was isolated from frozen cell pellets using the mirVANA miRNA Isolation kit (Ambion, Cat# AM1560) following the manufacturer’s instructions. The purified RNA samples typically derived from a maximum of $5 \times 10^6$ cells kept as frozen pellets using the AllPrep DNA/RNA Mini Kit (Qiagen, Cat# 80204) by following the manufacturer’s instructions. tRNApYl, PylRS, β2M mRNA expression levels were measured using TaqMan technology on the 7900HT Fast Real-Time PCR System (Life Technologies). The probes and primers were generated by assays-by-design (Applied Biosystems); the probes contain a 6-carboxy-fluoresceinphosphoramidite (FAM dye) label at the 5’end of the oligo and non-fluorescent (NFQ) or fluorescent quencher tetramethylrhodamine (TAMRA) at the 3’ end. qPCR was carried out by denaturing at 95°C for 20 s and then cycling at 95°C for 1 s and 60°C for 20 s, for 40 cycles. Primers for CHO-K1 β2M and 18S ribosomal RNA (18S) (Applied Biosystems, Cat# Hs99999901 s1) were used as the endogenous controls. Expression levels of tRNApYl processed and unprocessed and PylRS were normalized to endogenous CHO-K1 β2M or 18S. The sequence of the primers and probes are listed in Table 9.

### Western blot analysis

Total IgG from crude cell supernatants was quantified using Protein A biosensors (Pall ForteBio Cat# 18–5013), 15 ng of which was resolved on SDS-PAGE (BioRad, Cat# 1656001) under non-reducing condition and transferred onto PVDF membrane (BioRad, Cat# 1620175) using a Trans-Blot Turbo transfer system (Bio-Rad, Cat# 1704150). Membranes were probed with horseradish peroxidase-conjugated goat anti-human IgG (Fab’2) (Abcam Cat# ab87422) at 1:3000 dilution at room temperature for 1 h followed by 3-times washing in Tris-buffered saline (TBS) (Lonza, Cat# 1706435). Membranes were incubated briefly with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Cat# 34078) followed by image analysis by ImageQuant LAS 4000 (GE Healthcare Bio-Sciences).

### IgG purification

Full-length nnAA containing antibodies were captured from filtered cell culture supernatants by affinity chromatography (CaptureSelect FcXL, Life Technologies, Cat# 194328050). Bound IgG was washed with phosphate-buffered saline (PBS) pH 7.2 (Life Technologies, Cat# 20012050) and eluted with 0.1 M glycine pH 3.1 (Sigma, Cat# 410225) followed by neutralization with 20% v/v 1.0 M Tris pH 8.0 (Sigma, Cat# 93363). The purity of the IgG was confirmed by SDS-PAGE under reducing and non-reducing conditions and dialyzed to PBS pH 7.2 for long-term storage.

### Octet-based assay for quantitation of full-length and total IgG

Full-length IgG quantitation was performed by bio-layer interferometry in the Octet RED96 System (PallForteBio). Streptavidin biosensors (PallForteBio, 18–5019) were soaked overnight at 4°C in mouse monoclonal H2 anti-human IgG Fc biotin (Abcam, ab99766) diluted to 5 µg/ml in PBS pH 7.2 (Thermo Fisher, 20012050). Excess unbound streptavidin was washed by equilibration of biosensors to the cell culture medium for 10 min at room temperature. Total IgG quantification was performed using Protein A biosensors (PallForteBio, Cat# 18–5013). Binding rate measurements were collected in the Octet device and quantified within the Octet software in reference to known concentrations of IgG standards.

### Peptide mapping analysis

Antibody samples (100 µg) were denatured and reduced at 37°C for 30 min with buffer (1.3 M guanidine hydrochloride; 8 M urea; 130 mM Tris [tris (hydroxymethyl) aminomethane], pH 8.0; 6.5 mM dithiothreitol) followed by cooling at room temperature and alkylation by 2-iodoaceticamide in the dark for 30 min. After alkylation, the samples were diluted 4 times (from 50 µL to 200 µL) with 100 mM Tris, pH 7.5, before digestion. Tryptic digests were performed at 37°C for 4 h and then quenched by the addition of 5 µl of 10% (vol/vol) trifluoroacetic acid (TFA). A Fusion Orbitrap mass spectrometer (Thermo Fisher Scientific) connected with an AQUITY ultra-performance liquid chromatograph (UPLC; Waters) was used for peptide map analysis. An AQUITY UPLC BEH300 C18 column (1.7 µm, 2.1 × 150 mm; Waters) was used for separation. The column temperature was held at 55°C. Mobile phase A was 0.02% TFA in water, and mobile phase B was 0.02% TFA in acetonitrile. Digested peptides were eluted from the column with a linear gradient of mobile phase B buffer from 5% to 40% B in 68.5 min then 40–95% B in 4.5 min and the chromatographic profile was monitored by using ultraviolet absorbance at 220 nm and mass spectrometry. Mass spectrometry data were processed by Biopharma Finder 2.0 (Thermo Fisher Scientific).
Intact-mass measurements

Intact-mass analysis was performed with a Waters SYNAPT G2-Si High Definition Mass Spectrometer in conjunction with a UPLC system (Waters). Purified antibody samples were diluted to 1 mg/mL in 100 mM Tris buffer at pH 7.6 and deglycosylated using PNGase F at 37°C overnight. The deglycosylated samples were desalted using reverse phase chromatography on a BEH C4 column (2.1 mm × 50 mm; Waters), using a mobile phase A composed of 0.1% formic acid (FA) and 0.01% TFA in water and a mobile phase B of 0.1% FA and 0.01% TFA in acetonitrile. Samples were eluted on a linear gradient from 5% to 80% B in 2 min, then 80% to 95% B in 2 min, and mass spectra were collected at m/z range of 800–4,500. Molecular mass was determined by deconvolution with the Maxent I software package (Waters, Milford, MA).

3L Perfusion bioreactors

Cells were cultured in 3 L stirred tank bioreactors using Eppendorf’s DASGIP parallel bioreactor system. Bioreactors were inoculated from shake flask cultures to a working volume of 1.2 L and a starting cell density of 1.0 × 10^6 viable cells/mL using proprietary media. Operating parameters were monitored and controlled using DASware control software. Culture temperature, pH, and dissolved oxygen were maintained at 35.5°C, 7.00 ± 0.20, and 30%, respectively. The agitation was controlled at 350 rpm. Cell density and viability were measured using a Beckman Coulter Vi-CELL XR cell viability analyzer. Glucose, lactate, electrolytes, pH, pCO₂, and pO₂ were measured using a Siemens RAPIDPoint® 500 blood gas analyzer. Perfusion was initiated by alternating tangential flow filtration on day two of cultivation using proprietary enriched perfusion media. The culture was pumped back and forth through a Spectrum Labs Minikros Sampler hollow fiber module (P/N S04-P20U-10-N, polyethersulfone, 0.2 micron, 980 cm²). Replicien’s XCell™ ATF C24 controller was used to control the recirculation rate at 0.4 L/min. Initial perfusion rate was set to five vessel volumes per day (VVD) and adjusted up to 4 VVD based on viable cell density measurements. nnAA was supplemented to the bioreactor culture and perfusion media on day 5 at a final concentration of 2 mM.

Azide-alkyne conjugation

Purified antibody containing AzK in PBS pH 7.2 (Life Technologies, Cat# 20012050) was incubated overnight with tubulysin-DBCO (eight-fold molar excess) at 20°C in the presence of 5 mM sodium phosphate monobasic (Sigma Catalog, Cat# S0751–100G) and 10% v/v dimethyl sulfoxide (Sigma Catalog, Cat# D1435–500 mL). Conjugated material was captured with CHT II resin (BioRad, Cat# 158–2200) and washed promptly with 10 mM NaPO₄ pH 7.0 (Sigma, Cat# P2070). 2.0 M sodium chloride (J.T. Baker, Cat# 3624–19) was used to elute the conjugated mAb. Conjugation and purity were confirmed by SDS-PAGE and mass spectrometry.

Diels-Alder conjugation

Purified antibody containing CpK or SCpK in PBS pH 7.2 (Life Technologies, Cat# 20012050) was incubated for an hour with a maleimide-bearing payload (fivefold molar excess) at room temperature in presence of 10% NaPO₄ monobasic, pH 5.5 followed by quenching with four molar equivalents (relative to maleimide) of N-acetyl cysteine. Conjugated material was purified using CHT II resin (BioRad, Cat# 158–2200). Conjugation and purity were confirmed by SDS-PAGE and mass spectrometry.

Cytotoxicity assay

HER2-positive SK-BR-3 (ATCC, Cat# HTB-30), and HER2-negative MDA-MB-468 (ATCC, Cat# HTB-132) cells cultured in RPMI 1640 medium (Life Technologies, Cat# 20012050) supplemented with 10% fetal bovine serum were seeded at a density of 4,000 cells/well in 96-well white clear-bottom polystyrene microplates (Corning, Cat# 07–200–587) in 75 μL volume and allowed to grow overnight in humidified incubator at 37°C in presence of 5% CO₂. ADCs in PBS pH 7.2 (Life Technologies, Cat# 20012050) were serially diluted and added to each well with cells and incubated for 96 h. The viability of the treated cells was determined by adding CellTiter-Glo (Promega Corporation, Cat# G9242) and luminescence monitored using Perkin Elmer Envision Manager Software v8. A non-linear regression model was used to fit the data for each sample and the half-maximal effective concentrations (EC50) calculated using GraphPad Prism software.

Localization of PylRS and confocal imaging

Actively growing cells were fixed in a freshly prepared 1:1 mixture of methanol and acetone for 1 min at room temperature followed by washing with 1x-D-PBS (Invitrogen, Cat# 14190144) in a microfuge tube. The fixed cells were incubated at room temperature for an hour with 10 μg of APC-conjugated anti-FLAG M2 antibody (abcam, Cat# ab72569) in 100 μL of 3% BSA-PBS-T (TWEEN 0.1%) followed by an additional 30 min incubation with Alexa-488 labeled anti-α1 sodium potassium ATPase antibody at 1:100 dilution (abcam Cat# ab197496). The excess antibodies were thoroughly washed with 1x PBS-T (TWEEN 0.1%). Stained cells were mounted on positively charged slides in presence of DAPI containing anti-fade mounting agent ProLong Gold Antifade Mountant (ThermoFisher, Cat# P36930), then visualized and imaged by confocal microscopy using a Leica SP5 (Leica Microsystems Inc.).

Abbreviations

ADC antibody-drug conjugates
PyRS pyrrolysyl-tRNA synthetase
IgG immunoglobulin G
CHO Chinese hamster ovary
nnAA non-natural amino acids
tRNA transfer ribo-nucleic acid
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Disclosure of potential conflicts of interest

No potential conflicts of interests

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