The Pfenex Platform for Preclinical V_HH-ADC Development

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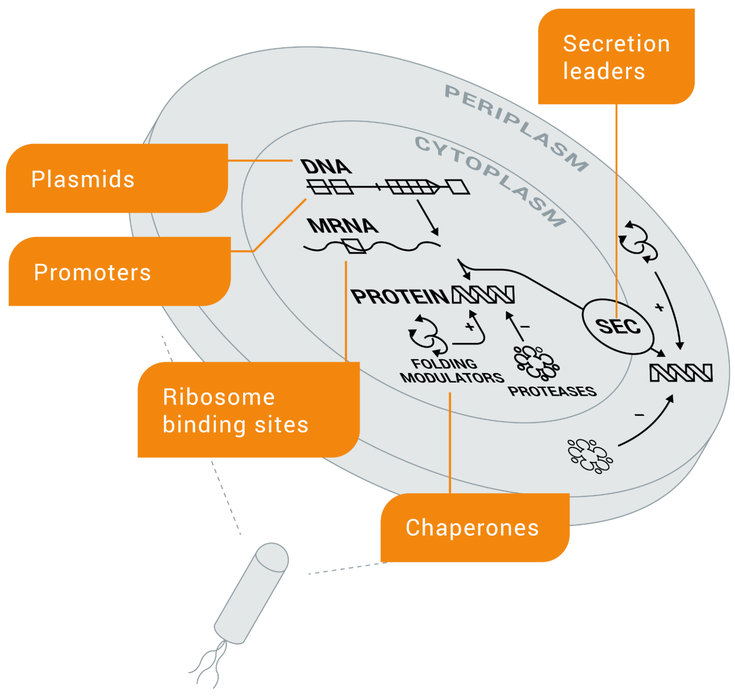
A V_HH therapeutic candidate with a single non-canonical amino acid substitution was produced in fermentation at comparable titers to the canonical version (~15 g/L)

ABSTRACT

Amber suppression technology has matured over the past 25 years, enabling incorporation of non-canonical amino acids (ncAAs) for site-specific conjugation and expanded therapeutic functionality. However, implementation has been limited by toxicity from off-target ncAA incorporation and substantially reduced protein yields at fermentation scale. We adapted an orthogonal aminoacyl-tRNA synthetase/tRNA pair for *Pseudomonas fluorescens*, the basis of the Pfenex Expression Technology® platform, to overcome these limitations. The system successfully incorporated para-azidophenylalanine (pAzF) into a novel anti-GPCR V_HH candidate while maintaining production titers of approximately 15 g/L at 2 L high-cell-density fermentation scale, comparable to its canonical counterpart. This approach preserves production economics while improving conjugation specificity and expanding protein functionality. We describe the identification of suitable conjugation sites, host-strain engineering, and scale-up to support preclinical *in vivo* evaluation.

BACKGROUND

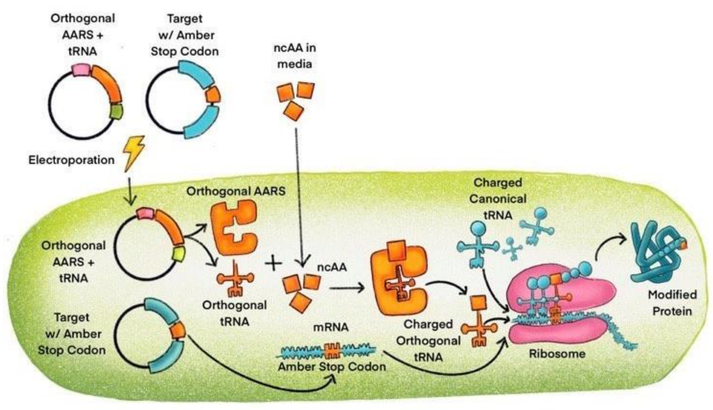
Rapid Strain Construction, Expression and Analysis:



- Bioinformatics and transcriptomics leveraged to develop an extensive toolbox of expression strains and plasmids
- Pfenx toolbox components are combined to produce hundreds or thousands of unique expression strains.

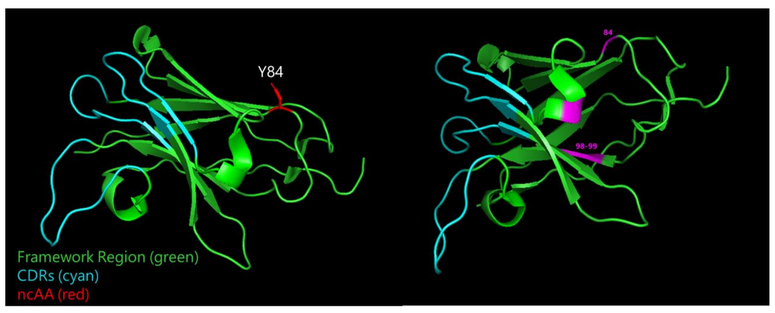
METHODS

Incorporation of an orthogonal amino acyl-tRNA synthetase/tRNA pair in *P. fluorescens*



V_HH substitution site selection

- Avoided side chains facing hydrophobic centers
- Avoided positions that may interfere with antigen binding
- Favored positions with lower steric hindrance for conjugation

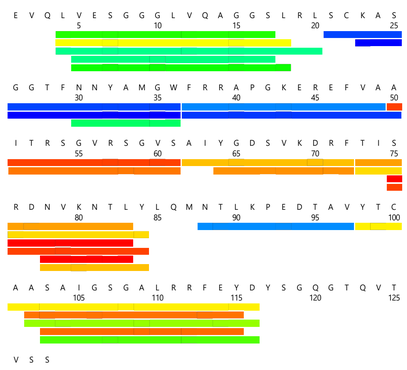


Selected substitutions for testing:
Y64pAzF, Y84pAzF, Y98pAzF, and T99pAzF

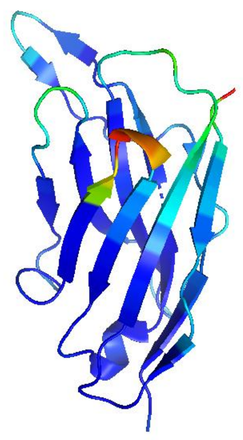
RESULTS

HDX Identified Solvent-Accessible Areas of the V_HH Fold for Site-Specific Conjugation

HDX solvent accessibility map of a V_HH protein, with highly exposed regions shown in red and buried regions in blue. HDX is a tool to aid in assessing amenable chemical conjugation site testing.



Sequence Coverage = 85.9%, Redundancy = 2.91



High Solvent Accessibility
Desirable for ncAA incorporation

Low Solvent Accessibility
Not desirable for ncAA incorporation

V_HH Substitution Site Testing in *P. fluorescens* using the Pfenex Toolbox

Plate-scale V_HH expression shows high soluble expression with 3 of 4 pAzF insertion sites tested

- Best ncAA performer produced 84% of the canonical (+) control strain
- Absence of soluble Y64pAzF V_HH likely due to ncAA side chain facing inwards towards hydrophobic center
- Selected Y84pAzF version for fermentation scale optimization based on good expression and good click reaction assessment

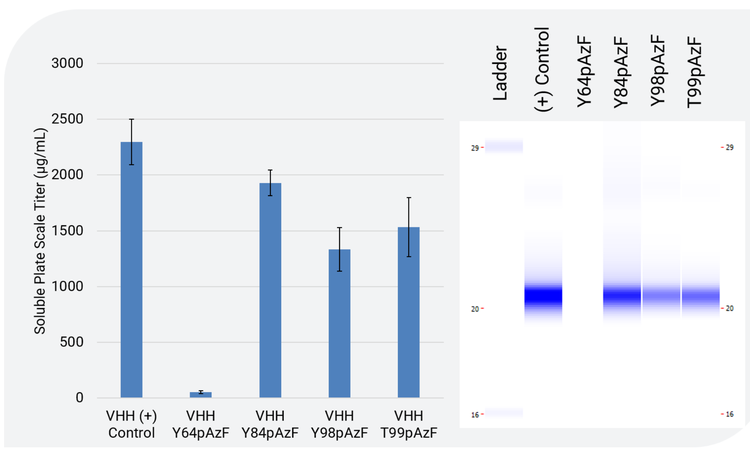


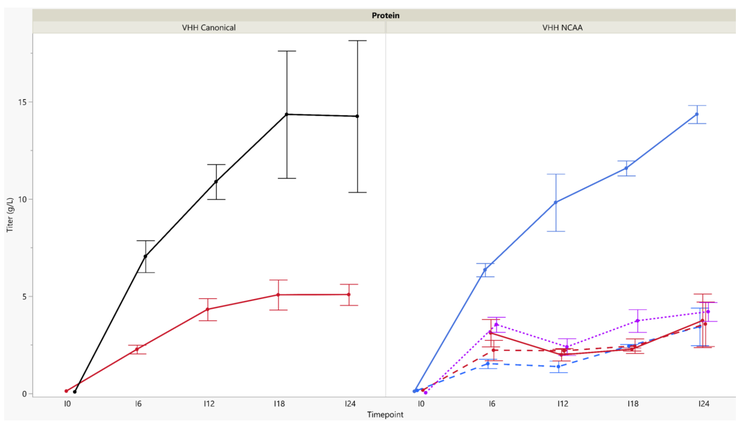
Plate scale ncAA V_HH testing: (Left) Soluble titers of V_HH from plate scale expression as determined by SDS-CGE (n=4). Of the substitutions that produced soluble V_HH, the production ranged from 58-84% of the canonical (+) control. (Right) Representative gel-like image generated from SDS-CGE.

Fermentation Optimization

How much pAzF should be added?

How should the carbon source (glycerol) be added?

How should pAzF be added?



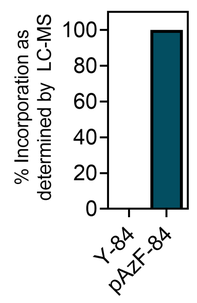
High Cell Density Fermentation: Titer assessed by SDS-CGE (triplicate samples) following V_HH expression induction.

Condition
- Continuous pAzF with continuous feed
- Continuous pAzF with DO stat
- High pAzF with continuous feed
- High pAzF with DO stat
- Low pAzF with continuous feed
- No pAzF with continuous feed

Fermentation Key Takeaways

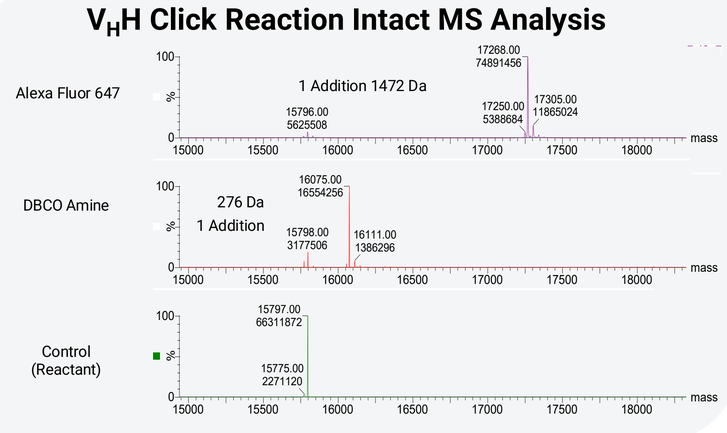
- High pAzF dosage had a negative impact on canonical V_HH yield
- Low pAzF bolus dosage at the time of induction, followed by a continuous pAzF feed enabled V_HH comparable to the canonical (+) control
- Low pAzF dosing with continuous feed improves pAzF utilization. Further optimization will improve ncAA utilization, balanced with target protein yield

Confirmation of ncAA incorporation

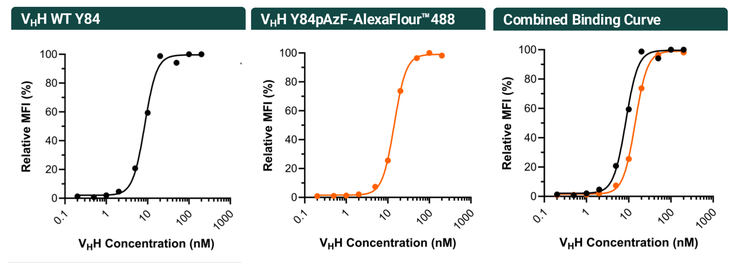


- 100% incorporation of pAzF and labeling (peptide map)
- Alkyne DBCO reagents (various sizes) 100% SPAAC (strain-promoted azide-alkyne click chemistry) converted at Y84pAzF
- WT Y84 control not reactive

V_HH Click Reaction Intact MS Analysis



Conjugation at Y84pAzF does not affect V_HH binding



Binding profiles of Y84 WT V_HH and conjugated Y84pAzF V_HH scaffold to target GPCR

- V_HH binding to the target GPCR receptor was confirmed by flow cytometry for both wild-type Y84 protein and the Y84pAz conjugated to Alexa Fluor™ 488
- Wild-type Y84 protein and SPAAC conjugated Y84pAzF-Alexa Fluor™ 488 scaffold had comparable binding affinity values of 8.5 and 14.2 nM, respectively

CONCLUSIONS

An amber suppression system for incorporating pAzF into recombinant proteins was adapted to *Pseudomonas fluorescens*, the expression host of the Pfenex platform. In addition, production of a single pAzF substituted V_HH therapeutic candidate was demonstrated in this host strain. Expression was optimized at fermentation scale: production closely mirrored canonical V_HH titer (~15 g/L) and pAzF utilization efficiency was increased to 46%. The ability to conjugate via pAzF was confirmed and conjugated V_HH was shown to maintain binding activity. Next steps are to generate the drug conjugate candidate and perform further *in vitro* and *in vivo* analysis. These results demonstrate the robust capabilities of the Pfenex platform and how it can help overcome some of the major hurdles to commercialization and scale-up of genetic code expansion technology in therapeutic proteins.

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