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Exploiting Single Domain Antibodies as Regulatory Parts to Modulate Monoterpenoid Production in *E. coli*

Jonathan Wilkes, Anthony Scott-Tucker, Mike Wright, Tom Crabbe, and Nigel S. Scrutton*

**ABSTRACT:** Synthetic biology and metabolic engineering offer potentially green and attractive routes to the production of high value compounds. The provision of high-quality parts and pathways is crucial in enabling the biosynthesis of chemicals using synthetic biology. While a number of regulatory parts that provide control at the transcriptional and translational level have been developed, relatively few exist at the protein level. Single domain antibodies (sdAb) such as camelid heavy chain variable fragments (V_HH) possess binding characteristics which could be exploited for their development and use as novel parts for regulating metabolic pathways at the protein level in microbial cell factories. Here, a platform for the use of V_HH as tools in *Escherichia coli* is developed and subsequently used to modulate linalool production in *E. coli*. The coproduction of a Design of Experiments (DoE) optimized pbB8k His(V_HH)CyDisCo system alongside a heterologous linalool production pathway facilitated the identification of anti-bLinS V_HH that functioned as modulators of bLinS. This resulted in altered product profiles and significant variation in the titers of linalool, geraniol, nerolidol, and indole obtained. The ability to alter the production levels of high value terpenoids, such as linalool, in a tunable manner at the protein level could represent a significant step forward for the development of improved microbial cell factories. This study serves as a proof of principle indicating that V_HH can be used to modulate enzyme activity in engineered pathways within *E. coli*. Given their almost limitless binding potential, we posit that single domain antibodies could emerge as powerful regulatory parts in synthetic biology applications.

**KEYWORDS:** single domain antibodies, design of experiments, monoterpenes, enzyme modulators, synthetic biology

The use of single domain antibodies (sdAb) as novel regulatory parts is an interesting yet unexplored opportunity in synthetic biology workflows for chemicals production. The often fine-specificities and high affinities of antibodies for their binding partners and the availability of display technologies to select for these properties make them attractive molecules for the in-cell targeting of proteins, large molecules, or other structures of interest. Antibodies, and fragments thereof, have been widely exploited as therapeutics, diagnostics, and imaging reagents. When considering the use of antibody fragments within the cell, targeted protein knockdown, novel transcriptional control, and targeted protein sequestration and labeling applications have been developed in mammalian, fish, and insect cells lines. However, to our knowledge, the use of sdAb to modulate metabolic pathways (e.g., for chemicals production) within microbial chassis has not been reported. In principle, sdAb binding could lead to multiple outcomes: these could include activation or inhibition of pathway enzymes; stabilization of enzymes; interception of small molecule regulation (e.g., allosteric regulation) or reprofiling of catalytic properties (e.g., product outcomes). sdAb binders possessing one or more of these characteristics may subsequently be exploited as novel regulatory parts that

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facilitate improved metabolic control at the protein level within microbial cell factories.

One of the most significant barriers to the development of antibody fragments for use in E. coli and other prokaryotic hosts is the inability of these chassis to carry out post-translational modifications required for function. While the use of sdAb bypasses any requirement for N-glycosylation, the formation of disulfide bonds remains necessary to ensure correct folding of the antibody and retention of function. Alternative strategies for the development of binding proteins as regulatory components include the use of recombinant binding proteins such as designed ankyrin repeat proteins (DARPins), monobodies, affibodies, anticalins, and affimers. The use of such scaffolds is advantageous as they do not require the formation of disulfide bonds, while large libraries of each can be constructed and subsequently enriched in the same manner as antibody libraries. However, sdAb such as camelid heavy chain variable fragments (VHH) possess an elongated binding loop (complementarity determining region 3), when compared to those of conventional antibody and recombinant binding protein formats. This facilitates binding of “cryptic” epitopes on the target antigen, such as those that occur within clefts and cavities, significantly expanding the potential application space of sdAb. The availability of specialized E. coli strains—for example those bearing gor/trxB reductase knockouts, and plasmid encoded systems such as the CyDisCo catalysts—facilitates bacterial production of antibody fragments in the cytosol and thus allows their use as regulatory parts.

Microbial cell factories for terpenoid production are attractive targets for in-cell modulation using sdAb. Terpenoids form one of the largest and most diverse classes of natural products, with over 80,000 structures reported, and many terpenoids have significant commercial and therapeutic value. The sesquiterpenoid artemisinin has antimalarial properties, with over 80,000 structures reported, and many attractive targets for in-cell modulation using sdAb. Terpenoids thus allows their use as regulatory parts.

To date, a variety of terpenoids have been produced (e.g., linalool synthase (bLinS) in E. coli isolated following the third and fifth injections were of size 1–5 × 10^7, respectively. Both libraries were enriched using biopanning utilizing two varying wash conditions, in an effort to capture a wider spectrum of bLinS binders. Following the first round of biopanning for each library and wash condition, 95 colonies from each were screened to determine the efficacy of enrichment. Of the 380 total colonies screened, 93% were identified as bLinS binders by ELISA. Sequencing of these clones yielded 261 unique VHH sequences and 158 unique CDR3 sequences within the enriched populations (Supplementary Figure 1).

**RESULTS AND DISCUSSION**

**Construction and Enrichment of anti-bLinS VHH Phage Display Libraries.** Two immune bLinS VHH phage display libraries were constructed following immunization of a llama with purified S. clavuligerus bLinS. A “Bleed 1” library and a “Bleed 2” library, constructed using genetic material isolated following the third and fifth injections were of size 1–5 × 10^7, respectively. Both libraries were enriched using biopanning utilizing two varying wash conditions, in an effort to capture a wider spectrum of bLinS binding affinities. Following the first round of biopanning for each library and wash condition, 95 colonies from each were screened to determine the efficacy of enrichment. Of the 380 total colonies screened, 93% were identified as bLinS binders by ELISA. Sequencing of these clones yielded 261 unique VHH sequences and 158 unique CDR3 sequences within the enriched populations (Supplementary Figure 1).

**DoE Optimization of Cytoplasmic VHH Production.** To utilize the resultant anti-bLinS VHH for synthetic biology applications, a platform that facilitated functional production of correctly folded antibodies within the cytoplasm of E. coli was
required. Given the large number of bLinS binders obtained following library enrichment, a series of optimized conditions for a single V_{HH} were first identified. Two of these platforms were subsequently tested on a wider subset of V_{HH} in order to determine the efficacy of each. As the most commonly occurring clone within the sequenced population of the enriched libraries, the anti-bLinS V_{HH} B2D C10 was chosen for the initial development of the production system.

When considering the optimization of protein production, traditional experimental approaches such as the sequential alteration of individual factors do not always prove time and resource effective. Furthermore, in large multifactor systems, key interactions between factors may be missed, resulting in an incomplete understanding of the system in question. This is especially true within biological systems in which complex interactions, such as those between environmental and genetic factors, may have significant effects on a chosen response. Design of Experiments (DoE) is a structured method of experimental design that provides statistical insights to better understand and subsequently optimize complex, multifactor systems. DoE has successfully been used within biological systems in order to explore changes in substrate specificity caused by amino acid substitutions, to understand and optimize metabolic pathways for the production of various metabolites, and for the evaluation and development of improved translational riboswitches.

Input variables for the experimental design were chosen based on both analysis of the literature and on previously conducted experimental pilot studies (data not included). When evaluating production strains to be used in this work, the inclusion of the Δgor ΔtrxB double knockout SHuffle T7 Express cells was considered necessary. In an effort to understand the effect of the double knockout system on disulfide bond formation, the regular T7 Express strain, with reducing pathways intact, was also included in the experimental design. Additionally, NiCo21 (DE3) (New England Biolabs) and NEB 10β (New England Biolabs) were also included for comparison. The second input variable included in the design was the coproduction of the CdDisCo catalysts yeast sulhydryl oxidase, Ervlp, and mature human PDI, which were developed for use as an alternative production strategy to Δgor ΔtrxB double knockout strains. The final categorical input variable explored was the addition of N-terminal tags to aid in protein solubility. When considering the small size of V_{HH} (~15 kDa), and that crystallographic data shows that the N-terminus of the polypeptide chain is adjacent to the CDR loops, the use of large solubility tags have the potential to interfere with the antibody–antigen interaction. Therefore, a series of short, flexible tags; His8 (15 amino acids), His8-TEV (28 amino acids), and His8 S-tag (46 amino acids), were investigated for their ability to improve production of functional B2D C10 (see Supplementary Table 1 for N-terminal tag amino acid sequences). Finally, inducer concentration and postinduction temperature were included in the design as continuous variables. Both factors are routinely varied at hoc when looking to optimize protein production in an effort to maximize the rate of protein synthesis while avoiding protein aggregation. The factor levels for each input variable used in the experimental design can be seen in Table 1.

When considering a suitable output response for the experimental design it was decided that measuring the total amount of V_{HH} produced would not provide an accurate representation of whether the protein had folded correctly and demonstrated the expected functionality. In an effort to determine the presence of functionally produced V_{HH} ELISAs using serially diluted cell lysate and NeutrAvidin captured biotinylated bLinS were performed. The absorbance measured at 630 nm minus the absorbance at 490 nm (ΔA_{630–490}) was used as a means of comparing the amounts of functionally active B2D C10 present.

**PLS Model Building and Interpretation.** Following execution of the experimental design, the mean ΔA_{630–490} values (n = 3) were calculated (Supplementary Table 2). The average ΔA_{630–490} values obtained using lysate at a 1:1000 dilution were then used to construct a partial least-squares (PLS) regression model using each of the effects included in the custom design. Model validation was performed using leave-one-out cross validation (LOOCV) and the statistically inspired modification of the PLS method (SIMPLS) algorithm. The optimal number of factors (latent variables) within each model was determined by choosing the fit which minimized the RM-PRESS statistic. For the first iteration of the PLS model, which included all main effects, all interactions, and powered terms for continuous variables, it was determined that two factors were optimal (RM-PRESS = 0.866). While a cumulative R^2 value of 0.899 indicated that the model explained a significant amount of variation in the Y variables, the predictive ability of the model, as determined by the cumulative Q^2 value of 0.437, suggested that the model could be further improved. Of the 67 terms included in the initial model 34 were identified as influential, as determined by the term having a variable importance in projection (VIP) statistic greater than 0.8.

To improve the predictive ability of the PLS model each of the terms with a VIP statistic greater than 0.8 (Figure 2A), including any nongsignificant main effect terms contained within significant interaction terms, were taken forward to construct a second PLS model. The resultant model, which utilized three factors (RM-PRESS = 0.789), demonstrated improved predictive power as exemplified by a cumulative R^2Y value of 0.929 and a cumulative Q^2 value of 0.702. A series of diagnostic plots were generated to further demonstrate the model was of sufficient quality for predictive use (Supplementary Figure 2). The actual by predicted plot (Supplementary Figure 2A) confirmed that the data were largely well predicted by the model. While the residual by predicted plot (Supplementary Figure 2B), residual by row (Supplementary Figure 2C), and residual normal quantile (Supplementary Figure 2D) showed a balanced distribution, with the exception of the edge effect seen in the residual by predicted plot (Supplementary Figure 2B) for experiments which had ΔA_{630–490} Values of 0, that is, no bound V_{HH} was detected.

### Table 1. Input Variables and Associated Factor Levels Chosen for This Experimental Design

<table>
<thead>
<tr>
<th>input variable</th>
<th>role</th>
<th>factor level</th>
</tr>
</thead>
<tbody>
<tr>
<td>strain</td>
<td>categorical</td>
<td>NEB10β, NiCo21 (DE3), T7 Express, SHuffle T7 Express</td>
</tr>
<tr>
<td>CyDsCo</td>
<td>categorical</td>
<td>NO, YES</td>
</tr>
<tr>
<td>N-terminal Tag</td>
<td>categorical</td>
<td>none, His8, His8-TEV, His8 S-Tag</td>
</tr>
<tr>
<td>inducer concentration (mM)</td>
<td>continuous</td>
<td>0.1–50</td>
</tr>
<tr>
<td>postinduction temp (°C)</td>
<td>continuous</td>
<td>16–30</td>
</tr>
</tbody>
</table>
To identify a combination of the input factors that yielded optimal functional B2D C10 production, the predictive power of the PLS model was used. The resultant prediction profiler plots showed the combination of factors that yielded the most desirable \( \Delta A_{630-490} \) (1:1000) output, which were assigned a desirability score between 0 and 1 (Figure 2B,C). The most desirable factor combination, that is, the combination with the highest predicted \( \Delta A_{630-490} \) (1:1,000) value had a score of 0.928. This combination utilized an N-terminal His\(_6\) tag, the coproduction of the CyDisCo catalysts and the use of SHuffle T7 Express cells, with induction using 50 mM arabinose and a reduction of the incubation temperature to 23.7 °C following induction (Figure 2B). When considering optimal production levels in alternative strains, the addition of an N-terminal His\(_6\) tag, and the coproduction of the CyDisCo catalysts in NEB 10β following induction with 50 mM arabinose and incubation at 26.6 °C, had the next highest desirability score of 0.874 (Figure 2C).

**Evaluating the Production of anti-bLinS V\(_{HH}\) Using Optimized Conditions.** Following the successful identification of two conditions that could be used to improve the cytoplasmic production of the anti bLinS V\(_{HH}\) B2D C10, both were tested for their ability to produce other anti-bLinS V\(_{HH}\). A selection of 24 anti-bLinS V\(_{HH}\) possessing the most commonly
occurring CDR3 sequences were chosen for further evaluation of the cytoplasmic production platform (see Supplementary Table 3 for aligned sequences). These VHH were cloned into the pBbE8k His6-RFP CyDisCo vector and produced using the optimized conditions described above. The cell lysate was then used to perform an ELISA against immobilized biotinylated bLinS in order to identify functional production of the VHH in question. When using the optimized conditions functional production of 21 out of 24 (87.5%) anti-bLinS VHH, as determined by a $\Delta A_{630-490}$ value 3-fold greater than that of the pBbE8k His-RFP lysate control, was observed (Figure 3). In comparison, when the same anti-bLinS VHH were produced in the absence of an N-terminal His 6 tag and CyDisCo catalysts only 5 of the 24 VHH selected (20.1%) were functionally produced (Supplementary Table 4). These results suggest that the optimized conditions identified herein represent a suitable platform upon which VHH may be used as binders within the cytosol of E. coli.

### Strain Comparison of In Vivo Linalool Production

During the optimization of anti-bLinS VHH production in E. coli using a DoE methodology, the most suitable strains were found to be SHuffle T7 Express and NEB 10\(\beta\). Given that the choice of strain, carbon source, and redox balance within the cell have been shown to affect limonene production,\(^{13}\) the ability of SHuffle T7 Express and NEB 10\(\beta\) cells to produce linalool using the pJBEI-6410 bLinS plasmid (Figure 4A) was first investigated in order to establish which strain was better suited for linalool production in the absence of anti-bLinS VHH. The strains NEB5\(\alpha\), BL21 (DE3), and T7 Express were also included for comparison.

Of the strains evaluated, NEB 10\(\beta\) produced the greatest quantity of linalool (Figure 4B). The use of 1% (v/v) glycerol as the primary carbon source facilitated the production of 87.1 ± 6.1 mg L\(_{org}\)\(^{-1}\) of linalool, while 1% (v/v) glucose resulted in 82.8 ± 35.4 mg L\(_{org}\)\(^{-1}\). SHuffle T7 Express produced the least linalool of the strains evaluated, achieving linalool titers of 0.2 ± 0.1 mg L\(_{org}\)\(^{-1}\) and 0.2 ± 0.3 mg L\(_{org}\)\(^{-1}\) when utilizing glucose and glycerol (1% v/v) as the carbon source, respectively. The relatively poor linalool titers produced by SHuffle T7 Express may be due to the altered redox pathways present in this strain.\(^{13}\)

As NEB 10\(\beta\) was a suitable production host for anti-bLinS VHH and also achieved the highest linalool titers under the conditions tested, this strain was the obvious choice to carry forward for future experiments, with glycerol as the primary carbon source. To evaluate the modulatory effects of anti-bLinS VHH on the production of linalool, conditions that were developed to favor the production of anti-bLinS VHH (His\(_6\) tag, CyDisCo catalysts, NEB 10\(\beta\), 50 mM arabinose, and 26.6 °C postinduction temperature) were taken forward for in vivo experiments.

### In Vivo Modulation of bLinS Using anti-bLinS VHH

During initial efforts to produce anti-bLinS VHH alongside the heterologous linalool production pathway, upon induction of cells transformed with both pJBEI-6410 bLinS and pBbE8k His\(_6\)-VHH CyDisCo, cell growth was severely impaired, preventing reliable analysis of the terpenoid products. This might reflect an increased metabolic burden as a result of VHH overproduction. Of the factors investigated during the DoE guided optimization of B2D C10 production in NEB 10\(\beta\), it was noted that inducer concentration had a less significant impact on the Prediction Profiler desirability score than altering the choice of N-terminal tag or removing the CyDisCo catalysts. In an effort to reduce metabolic burden, the decision...
was made to reduce the amount of arabinose added for the induction of VHH production.

When the concentration of arabinose added was reduced to 2 mM, reliable growth of pJBEI-6410 bLinS and pBbE8k His6-VHH CyDisCo transformed NEB 10β was observed. Furthermore, altered linalool titers were observed in a number of cases when comparing the pJBEI-6410 bLinS only (135.5 ± 19.4 mg L⁻¹) and pJBEI-6410 bLinS + pBbE8k His6-RFP CyDisCo (143.8 ± 35.1 mg L⁻¹) controls. The coproduction of VHH B2D C10, B2D D12, B2M H9, B1M C12, and B2M F2 resulted in significantly reduced linalool titers, suggesting that these VHH function as inhibitors of bLinS in the cytoplasm. Coproduction of anti-bLinS VHH B1D E6, B2D D7, B2D H6, B2M B9, B1D G9, B2M B8, and B2D E6 facilitated increased linalool titers; with B2D D7 resulting in an approximate 2-fold increase in linalool production (284.5 ± 3.3 mg L⁻¹). This suggests that these VHH stabilize the enzyme/substrate complex within the cytoplasm.

In addition to the production of linalool, geraniol and nerolidol were also detected by GC-MS (Figure 4D,E). The production of geraniol by E. coli containing a heterologous MVA pathway and GPPS in the absence of a geraniol synthase gene was reported previously and identified to be a result of promiscuous activity of native enzymes including alkaline phosphatase (PhoA). In this investigation, increased geraniol titers were observed for each of the previously identified inhibitory VHH (Figure 4D). The increased geraniol production in vivo is likely a result of the changing flux within the biosynthetic pathway occurring in response to the VHH-bLinS binding. The inhibitory effect of the anti-bLinS VHH is likely to create a bottleneck within the pathway, resulting in the accumulation of GPP precursors IPP and DMAPP within the
cell, both of which have been shown to be cytotoxic in *E. coli*.\(^{35-37}\) As a result of increased GPP availability, conversion to geraniol by PhoA and other unidentified endogenous pathways\(^{21}\) would alleviate toxic effects of isoprenoid precursor accumulation (Figure 5).

*S. clavuligerus* bLinS has also been shown to have nerolidol synthase activity in the presence of the C15 sesquiterpenoid precursor farnesyl pyrophosphate (FPP).\(^{38}\) Strains of *E. coli* are capable of producing FPP using native enzymes.\(^{39}\) Nerolidol titers were reduced in the presence of each of the anti-bLinS VHH, with the notable exception of B2D E6 which instead conferred increased titers of nerolidol detected (Figure 4E). This could suggest that the majority of the VHH selected interact with bLinS near the active site, and thereby impair access of the longer C15 substrate. With the VHH B2D E6, however, the ability of bLinS to turn over both the C10 and C15 substrates was improved. The reduction of nerolidol titers was most significant in the presence of the VHH B2D C10, B2D D12, B2M H9, B1M C12, and B2M F2, when compared to the pBbE8k bLinS only and pBbE8k + pBbE8k-RFP CyDisCo controls, further suggesting that these VHH are indeed inhibitors of bLinS.

Finally, it was also observed that the titers of indole, an intracellular signaling compound that was identified previously as a potential inhibitor of isoprenoid pathway activity,\(^{40}\) were significantly reduced following the production of each anti-bLinS VHH as well as for the pBbE8k His\(_6\)-RFP CyDisCo control (Supplementary Figure 3). This suggests that the presence of the second plasmid and the coproduction of the CyDisCo catalysts may alleviate potential toxicity issues attributed to the accumulation of toxic isoprenoid precursors and monoterpenoids within the cell, and thus lower the observed indole titers. We propose that this could be due to a more gradual accumulation of isoprenoid precursors and monoterpenoids within the chassis, due to increased metabolic burden within cells bearing both plasmids, resulting in a muted isoprenoid stress response.

In this proof of principle study, we have demonstrated that VHH sdAb can be used as novel regulatory parts to modulate engineered metabolic pathways in *E. coli*. We have also demonstrated that a DoE workflow can be used to optimize expression of VHH binders in *E. coli*, which can be readily adapted for use with other microbial species. Using this workflow, and with continued development, the generation of sdAb with a variety of regulatory functionalities is feasible. This could open up new opportunities to regulate engineered pathways through controlled expression of highly specific VHH binders. Here, we have described inhibitors and activators of linalool production but other applications are also envisaged. For example, high affinity binders with no observed modulatory effect on bLinS could be used as a scaffold or tag to facilitate colocalization of enzymes. In monoterpene
producing pathways, GPPS and monoterpene synthases are known to be rate limiting enzymes, and their colocalization through tethering has been shown to increase the monoterpeneoid titers obtained. When considering applications of inhibitory binders, targeted knockdown of a variety of proteins have been explored in eukaryotic systems, demonstrating that sdAb are viable knockdown alternatives to gene editing technologies and RNAi based approaches. This work shows that targeted knockdown using sdAb is also possible in E. coli, facilitating the development of novel repression or feedback control systems with synthetic biology applications. Furthermore, the ability to alter pathway flux, as described here for the production of geraniol via native detoxification pathways following bLinS inhibition, is also significant. An improved ability to redirect metabolic pathways in an inducible, tunable manner provides a platform for the development of a new generation of microbial cell factories. As such, the VHH platforms and associated workflows described in this communication could now facilitate broad uptake of VHH antibodies as novel parts for metabolic pathways engineering and regulation.

METHODS

Vector Construction. All primers/oligonucleotide sequences used in this study can be found in Supplementary Table S. Purification of PCR products and agarose gel extractions were performed using a Nucleospin Gel and PCR Clean-up Kit (Macherey-Nagel). All constructs were sequenced by Eurofins Genomics to ensure correct sequence identity.

For the production of bLinS used in in vitro assays the codon optimized Streptomyces clavuligerus bLinS synthase (bLinS) gene was cloned between the NcoI and XhoI restriction sites of the pETM11 vector as described previously. The in vivo production of biotinylated bLinS was achieved using Lucigen’s Expresso Biotin Cloning and Expression System. S. clavuligerus bLinS from pETM11 bLinS was PCR amplified using Expresso bLinS Biotinylation Fw & Expresso bLinS Biotinylation Rv primers. Biotin XCell F’ chemotactic competent cells (Lucigen) were transformed with the purified PCR product and the linear pAviTag C-His vector. Colonies were screened by cPCR using pRham Fw and pETite Rv to determine the presence of the desired insert.

For the production of linalool in vivo, bLinS was cloned into the pJBEI-6410 vector. pJBEI-6410, a gift from Taek Soon Lee (Addgene plasmid #47049; http://n2t.net/addgene:47049), was PCR linearized using primers pJBEI-6410_Syn_open_3’ and pJBEI-6410_Syn_open_S’. Codon optimized bLinS was PCR amplified from pETM11 bLinS, using primers LinS+bLinS_Fw and LinS+bLinS_Rv. The purified PCR products were then cloned using an InFusion Cloning Kit (Clontech). Following the transformation of Stellar Competent cells (Clontech), cPCR using Syn_Seq_Fw and Syn_Seq_Rv primers was performed to confirm the presence of the insert. The resultant plasmid was subsequently referred to as pJBEI-6410 bLinS.

For the construction of vectors containing the CyDisCo catalyst system, codon optimized genes for the S. cerevisiae sulphhydryl oxidase Erv1p and mature human PDI were synthesized (Integrated DNA Technologies). The CyDisCo catalyst cassette included bespoke S’ UTR and RBS sequences designed using the Ribosome Binding Calculator and the rhaB promoter (coding sequences can be found in Supplementary Table 6). Initially, the synthesized CyDisCo fragment was cloned into the PCR linearized pBBE8k vector (pBBE8k CyDisCo Fw & Rv primers, Supplementary Table S) using an In-Fusion Cloning kit (Clontech). The resultant plasmid was subsequently referred to as pBBE8k RFP Rha-CyDisCo. For use in the DoE vectors the CyDisCo system was instead cloned into the pBBE8k vector under the control of the araBAD promoter, such that the RFP/VHH ErV1p, and PDI proteins were produced in a tricistronic manner. This was achieved by PCR amplification of pBBE8k RFP Rha-CyDisCo using pBBE8k Tri-Cistronic CyDisCo Fw and Rv primers. The purified PCR product was then phosphorylated and ligated using KLD Enzyme Mix (New England Biolabs), prior to transformation of NEB 10β competent cells. The resultant plasmid was referred to as pBBE8k RFP CyDisCo.

For the addition of N-terminal tags to the pBBE8k RFP CyDisCo expression vector, the plasmid was digested using the NdeI endonuclease in tandem with Antarctic phosphatase (New England Biolabs). N-terminal His12 His12-TEV and His8 S-tags with 15 bp overhangs were designed and synthesized (Integrated DNA Technologies) such that there was complementarity to the digested backbone and that the NdeI restriction site was restored at the 3’ end of the insert following cloning. In-Fusion Cloning (Clontech) was used to assemble the tagged RFP tricistronic CyDisCo expression cassette.

Select VHH coding sequences were PCR amplified using VHH NdeI Fw and VHH XhoI Rv primers. The purified VHH insert and pBBE8k tagged-RFP CyDisCo vector variants were digested using NdeI and XhoI endonucleases and subsequently gel purified using the NucleoSpin kit, prior to ligation using T4 DNA ligase (New England BioLabs). NEB 10β cells were transformed with the ligation mixture and plated on LB kanamycin (50 μg mL⁻¹) plates supplemented with 1 mM arabinose, such that instances of vector background could be more easily identified by the presence of red fluorescent protein producing colonies. cPCR using pBBE8k Seq Fw and Rv primers was performed to identify colonies containing the correct size of insert, which were subsequently sent for Sanger sequencing (Eurofins Genomics).

Protein Production. bLinS was produced and purified as described previously. Biotinylated bLinS was produced in vivo using Lucigen’s Expresso Biotin Cloning and Expression System. Following the construction of the pAviTag C-His bLinS vector, a single colony was used to inoculate small scale cultures of LB broth (ForMedium) supplemented with kanamycin (30 μg mL⁻¹), which were incubated at 37 °C overnight. These were used to inoculate (1:100) 6 × 1 L cultures which were grown in LB broth (ForMedium) containing kanamycin (30 μg mL⁻¹) and incubated at 37 °C with 190 rpm shaking, until an OD₆₀₀ of 0.3 was reached. Recombinant protein production was induced by the addition of 20% (w/v) rhamnose, 10% (w/v) arabinose, and 5 mM biotin solutions, to obtain final concentrations of 0.2% (w/v), 0.01% (w/v), and 50 μM, respectively. After 24 h the cells were harvested by centrifugation at 6000g, 4 °C, for 10 min, and the pellet was resuspended in Buffer A supplemented with DNase (0.1 mg mL⁻¹), lysozyme (0.1 mg mL⁻¹), and an EDTA free protease inhibitor tablet. The harvested cells were then lysed and purified as described by Karuppiah et al. (2017).

For the production of anti-bLinS VHH, the appropriately chemically competent cells were transformed with the pBBE8k His₈-VHH CyDisCo variants. Single colonies were used to...
inoculate 2 mL of LB overnight cultures supplemented with kanamycin (50 μg mL\(^{-1}\)). These initial cultures were incubated overnight at 30 °C with shaking at 200 rpm and used to inoculate TB supplemented with kanamycin (50 μg mL\(^{-1}\)) as a 1:100 dilution. The cultures were grown at 30 °C with shaking (200 rpm) until an OD\(_{600}\) of 0.6 was reached. The cultures were then induced by the addition of arabinose to a final concentration of 50 mM, and the temperature was subsequently altered to 26.6 °C. The induced cultures were then incubated for a further 24 h, before centrifugation and removal of the supernatant. Cell pellets were then flash frozen in liquid nitrogen and stored at −80 °C or immediately taken forward for cell lysis. Lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5% (v/v) glycerol, 0.1 mg mL\(^{-1}\) DNase, and 0.1 mg mL\(^{-1}\) lysozyme) was added to the frozen pellet at 1/10th of the initial culture volume, and the cell pellet was resuspended by vortexing. Three additional freeze-thaw cycles were performed to ensure thorough lysis of cells. The lysate was then clarified by centrifugation at 13000g for 10 min, and the supernatant was removed and retained for further analysis/purification.

Protein purity was monitored by SDS-PAGE. Protein concentration was determined using both the Bradford protein assay (Bio-Rad) and extinction coefficient methodology.

**Construction of anti-bLinS Phage Display Library.** A single male llama (*Llama glama*) was provided for use by UCB as part of their collaboration with the University of Reading. All llama handling steps were performed by a trained expert according to protocols compliant with the Animal (Scientific Procedures) Act 1986. The llama was given five subcutaneous injections of purified *Streptomyces clavuligerus* linalool synthase (bLinS) at monthly intervals, with bleeds (∼500 mL) taken after the third (Bleed 1) and fifth (Bleed 2) injections. bLinS (purified as described above) was prepared as five 500 μL aliquots (2 mg mL\(^{-1}\)) which were mixed 1:1 with GERBU adjuvant prior to immunization.

During the library construction process Bleed 1 and Bleed 2 reactions were performed independently, such that two libraries were obtained. B-cell mRNA was isolated from PBMC (Bleed 1:5 × 10\(^6\) cells, Bleed 2:3 × 10\(^6\) cells) using a Qiagen TissueRuptor and RNaseasy plus midi kit. mRNA isolated from each library was taken forward for RT-PCR using SuperScript IV reverse transcriptase (Invitrogen) with both oligo-dT and random hexamer (dN6) primers. Primary PCR amplification of the oligo-dT and dN6 generated cDNA between the signal peptide and CH2 domain of IgG/HCAb coding sequences was achieved using KOD Polymerase (Merck Millipore) with primers CaLL01 and CaLL02 (Supplementary Table S). The oligo dT and dN6 primary PCR products were pooled, and the V\(_{HH}\) population (600 bp) was purified. The purified V\(_{HH}\) amplicons were used as template for a secondary PCR using Herculase II polymerase (Agilent) with primers V\(_{HH}\)Sh Fw and CldlLib2Rv-01 or CldlLib2Rv-12, for Bleeds 1 and 2, respectively (Supplementary Table S). Secondary PCR products were purified and digested, alongside the phagemid vector (provided by UCB Pharma), using *NcoI* and *SfiI* endonucleases. Ligation of the digested phagemid vector (4 μg) and V\(_{HH}\) insert (1 μg) was performed using T4 DNA Ligase (New England Biolabs).

Electrocompetent TG-1 cells (Lucigen) were transformed with the purified ligation product. Following the recovery of cells, the transformation mixtures were pooled, and serial dilutions (10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\), 10\(^{-5}\)) of the recovered transformants were spread onto 2X YT Agar, 1% glucose, 100 μg mL\(^{-1}\) carbenicillin plates, and incubated overnight at 37 °C such that the library size could be estimated. The remaining transformation mixture was pelleted and resuspended in 1 mL of LB medium. The cell slurry was then spread across three XL Bio dishes (2X YT Agar, 1% glucose, 100 μg mL\(^{-1}\) carbenicillin) per library and incubated overnight at 30 °C. The following day each XL Bio dish was washed with 2X YT, 1% glucose, 100 μg mL\(^{-1}\) carbenicillin and the cell slurry collected. The slurry was used to inoculate a fresh culture of 2X YT, 1% glucose, 100 μg mL\(^{-1}\) carbenicillin at a starting OD\(_{600}\) of 0.1 and grown at 37 °C, with shaking (200 rpm), until an OD\(_{600}\) of 0.5 was reached. M13KO7 helper phage, at a multiplicity of infection (MOI) of 20, was used for library rescue as per established protocols. Phage particles were recovered from the culture supernatant by precipitation using 20% PEG 8000, 2.5 M NaCl. The purified phage were resuspended in PBS, 20% glycerol. Library size was calculated by phage infective titering.

**Enrichment of anti-bLinS Phage Display Library.** For each panning condition, sufficient phage particles were added so as to cover the estimated diversity of the libraries 20,000 fold. The phage libraries were blocked in an equal volume of 3% BSA, 3% milk powder in PBS for 1 h at room temperature with rolling. Simultaneously, NeutrAvidin coated Nunc Maxisorp 96 well plates (Invitrogen) or Dynabeads M-280 Streptavidin (Invitrogen) were blocked with 1% BSA, PBS for 1 h with shaking/rolling. Biotinylated bLinS (2 μg mL\(^{-1}\)) in 1% BSA, PBS was then added to the blocked libraries for each panning condition and incubated for 1 h. The biotinylated bLinS-phage mixture (and a phage only control) was subsequently added to the coated microtiter plate/Dynabead slurry and incubated for 1 h with gentle agitation. The coated microtiter plate/Dynabead slurry (Dynabeads were pelleted using a DynaMag-2 magnet) was then washed using PBS 0.1% Tween-20 (5 × 300 μL for microtiter plate and 10 × 2 mL for Dynabeads), followed by an additional two washes (300 μL and 2 mL for microtiter plate and Dynabeads respectively) using PBS. The bound phage particles were eluted by the addition of trypsin (1 μg mL\(^{-1}\) in PBS, 1 mM CaCl\(_2\)) which was added to the dried microtiter plate/Dynabeads and left to incubate at room temperature with shaking (600 rpm) for 30 min. The trypsin treated supernatant containing the eluted phage particles were then allowed to infect *E. coli* TG1 cells grown in 2X YT Broth (ForMedium) at an OD\(_{600}\) of 0.2–0.4. The infected TG1 cells were incubated (static) at 37 °C for 30 min. In order to estimate the output phage diversity, a serial dilution (10\(^{-4}\) to 10\(^{-5}\)) of the infected TG1 cells in 2X YT was performed, with the dilutions plated onto small LB agar plates supplemented with 1% glucose and 100 μg mL\(^{-1}\) carbenicillin. The output titers were subsequently estimated by counting the colonies present at each dilution after 16 h of incubation at 37 °C.

Small scale monoclonal phage rescue of individual colonies from the output phage titer plates obtained following library enrichment was performed using M13KO7 helper phage (New England Biolabs). Binding of enriched Bleed 1 and Bleed 2 monoclonal V\(_{HH}\) to biotinylated bLinS was confirmed by ELISA. Following the identification of biotinylated bLinS binders, fresh 96 deep-well culture blocks containing LB medium supplemented with 100 μg mL\(^{-1}\) carbenicillin and 2% glucose were inoculated using the initial monoclonal rescue blocks as a 1:100 inoculum. Following overnight growth the
phagemid vector was isolated using a 96-well plate plasmid DNA Miniprep kit (Bio Basic) and a 96-well plate vacuum manifold (Phenomenex). The phagemid vectors were sequenced using LMB3 and FDSEQ primers (Supplementary Table S), and the transcribed sequences were aligned using the CLUSTAL Omega sequence alignment tool. Sequence logos were created using the WebLogo server version 2.8.2.35

**Design of Experiments and PLS Regression Analysis.** Design of Experiments, statistical analysis and partial least-squares (PLS) regression was performed using JMP Pro 12 (SAS Institute). The Custom Design tool was used to create an I-optimal design that investigated all main effects (single effects of the variables themselves), two-factor interactions (interactions between two variables), and power terms for the continuous variables (an interaction of a continuous variable against itself in order to test for curvature). The experimental design consisted of a mixture of categorical and continuous input variables (experimental factors). The categorical variables used in the design were N-terminal tag (None, His6, His6 -TEV, and His6 S-tag), CyDisCo (No, Yes), and strain (NEB10β, NiCo21 (DE3), T7 Express, and SHuT7 Express). The continuous variables were arabinose concentration (0.1–50 mM) and postinduction temperature (16°C–30°C). The cytoplastic production of functional anti-LinS VHH was determined by ELISA; as such the absorbance at 630 nm minus the absorbance at 490 nm (∆A630−490) was used as the absorbance variable. The resultant design consisted of 42 experiments (Supplementary Table 2) which were performed in parallel in biological triplicate (n = 3). N-terminal tag and CyDisCo sequences can be found in Supplementary Tables 1 and 6, respectively.

Modeling of the experimental data was performed by PLSR. Model validation was performed using leave-one-out cross validation (LOOCV) and the SIMPLS (statistically inspired modification of the PLS method) algorithm. The root mean predicted residual error sum of squares (RM-PRESS) statistic was used to determine the optimal number of factors (latent variables) within the model. The variable importance in projection (VIP) and coefficients for each variable within the model were then used to identify those that had the greatest effect. Variables with a VIP > 0.8 were taken forward to fit a second iteration of the PLSR model which was validated using the methods described above.

**Enzyme-Linked Immunosorbent Assays (ELISA).** Wash steps utilized PBS 0.1% (v/v) Tween-20, while the incubation of the plates between steps was performed at room temperature for 1 h with shaking. Nunc Maxisorp 96-well plates were coated with 50 μL of NeutrAvidin or Streptavidin (1 μg mL−1 PBS) and incubated at 4°C overnight. The following day all plates were washed and blocked for 1 h at room temperature using 1% BSA in PBS. The blocked plates were again washed, and 50 μL of biotinylated bLinS (1 μg mL−1 in PBS, 5% (v/v) glycerol and 5 mM MgCl2) was added prior to an additional incubation period. After a further washing step, cell lysate was added in triplicate (n = 3). For titration ELISA, lysate was diluted in Buffer A prior to addition to the blocked plates. For monoclonal phage rescue ELISAs, supernatant containing monoclonal phage displaying anti-bLinS VHH was blocked in an equal volume of PBS 2% BSA 2% milk powder, prior to addition onto the biotinylated bLinS coated plates. Following addition of anti-bLinS VHH, the plates were incubated for 1 h before being washed. Bound VHH was revealed using MonoRab antimouse VHH HRP-conjugated monoclonal antibody (GenScript) at a 1:5000 dilution in PBS. Following the addition of 1 Step Ultra TMB ELISA Substrate (ThermoFisher) the absorbance at 630 and 490 nm was measured using a BioTek Synergy HT microplate reader. Antibody binding to the immobilized antigen was calculated by subtracting the absorbance at 490 nm from the absorbance at 630 nm (∆A630−490).

**Production of Monoterpenoids In Vivo.** For the production of monoterpenoids in vivo NEB 10β, NEB Stx, BL21 (DE3), T7 Express, and SHuT7 Express were transformed with pJBEI-6410 bLinS. Freshly transformed colonies were then used to inoculate LB (FortMedium) supplemented with 100 mg mL−1 carbenicillin, which were grown overnight at 30°C. Overnight cultures were used to inoculate (1:100 dilution) TB (FortMedium) supplemented with 1% (v/v) glucose or glycerol and 100 mg mL−1 carbenicillin in glass screw capped vials (2 mL reaction volume) which were incubated at 30°C with shaking at 200 rpm. Once an optical density (OD600) of 0.6 was reached linalool production was induced with 100 μM IPTG. Following induction, a 20% (v/v) n-nonane overlay was also added so as to capture the volatile monoterpenoids produced. The cultures were incubated for a further 72 h, before the organic layer was collected and dried using anhydrous MgSO4. The dried organic phase was then mixed at a 1:1 ratio with 1 mL of NeutrAvidin or Streptavidin coated plates. Following addition of 1 step Ultra TMB ELISA Substrate (ThermoFisher) the absorbance at 630 and 490 nm was measured using a BioTek Synergy HT microplate reader. Antibody binding to the immobilized antigen was calculated by subtracting the absorbance at 490 nm from the absorbance at 630 nm (∆A630−490).

**GC–MS analysis.** Samples were injected onto an Agilent Technologies 7890A GC coupled to an Agilent 5975A MSD. The products were separated using a DB-WAX column (30 m; 0.32 mm; 0.25 μm film thickness, JW Scientific). The injector temperature was 220°C with a split ratio of 50:1 (1 μL injection). The carrier gas was helium with a flow rate of 3 mL min−1 and a pressure of 8.3 psi. The oven program began at 40°C with a hold for 2 min followed by an increase of temperature to 70°C at a rate of 6°C/min; after this point the temperature was increased to 210°C at a rate of 50°C/min with a final hold at 210°C for 2 min. The ion source temperature of the mass spectrometer was set to 210°C, and spectra were recorded form m/z 50 to m/z 250. The mass spectra fragmentation patterns that were obtained were entered into the NIST mass spectral library for identification, product identity was also confirmed by the use of commercially bought standards. Quantification of the products was calculated using the ratios between the internal standard and the standard prepared at a known concentration.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00375.
Additional information relating to \( V_{HH} \) sequences obtained from immune libraries, DoE and PLSR model building, screening of optimized production conditions, and primers used in this work (PDF)

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**Author Contributions**

J.W., A.S.T., M.W., T.C., and N.S.S. wrote and reviewed the manuscript text. Figures were prepared by J.W. Phage display libraries were constructed by J.W. and A.S.T. using methods developed by A.S.T. and M.W. All remaining experimental work was proposed, designed, and performed by J.W.

**Notes**

The authors declare no competing financial interest.

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