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Increasing the secretory capacity of Saccharomyces cerevisiae for production of single-chain antibody fragments

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We have produced single-chain antibody fragments (scFv) in Saccharomyces cerevisiae at levels up to 20 mg/L in shake flask culture by a combination of expression level tuning and overexpression of folding assistants. Overexpression of the chaperone BiP or protein disulfide isomerase (PDI) increases secretion titers 2–8 fold for five scFvs. The increases occur for scFv expression levels ranging from low copy to ER-saturating overexpression. The disulfide isomerase activity of PDI, rather than its chaperone activity, is responsible for the secretion increases. A synergistic increase in scFv production occurs upon cooverexpression of BiP and PDI.

Keywords: expression system, yeast, BiP, PDI

Single-chain antibody fragments have a wide range of potential applications in biotechnology and medicine¹. Antibody fragments have been produced by a variety of methods with varying degrees of success²⁻⁸. The yeast Saccharomyces cerevisiae is an attractive host for expression of these heterologous disulfide bonded proteins, because the eukaryotic quality control mechanism of yeast ensures that all secreted protein is properly folded⁹. Disulfide-stabilized diabodies dimerize essentially completely when secreted from the yeast Pichia pastoris, whereas in E. coli, disulfide pairing was 10–70% for the same constructs¹⁰. Although the soluble fraction of antibody fragments expressed in the bacterial periplasm is generally functional, not all of the antibody protein secreted to the periplasm is soluble. Such is the case for McPC603 Fv¹¹ and 4-4-20 scFv, which is only 2% native when expressed in the E. coli periplasm¹². High cell density fermentation technology is well developed for S. cerevisiae as it is for E. coli. Unlike bacterial hosts such as E. coli, yeast is a generally recognized as safe (GRAS) organism free of pyrogens and infectious viruses. The most comprehensive genetic tool kit available for any yeast is an additional advantage of S. cerevisiae as an expression host. Presently, only expression of Fab fragments¹³ and full length, glycosylated IgM¹⁴ have been reported in S. cerevisiae; however, scFvs that lack potentially immunogenic N-linked glycosylation sites are well suited for S. *cerevisiae* production. In yeast systems, integrating versus episomal expression vectors can greatly affect secretion. The methylotrophic yeast, Pichia pastoris, can produce levels of heterologous proteins an order of magnitude greater than S. cerevisiae¹⁵. When chromosomal integration into the Ty δ sites of S. cerevisiae¹⁶ is used, allowing for expression level tuning by variable gene amplification, S. cerevisiae and P. pastoris produce very similar levels of bovine pancreatic trypsin inhibitor (BPTI)¹⁷. Even at optimized gene dosage, the potential to increase secretion through manipulation of the protein folding apparatus still exists. In particular, soluble levels of immunoglobulin heavy chain binding protein BiP/GRP78, a member of the HSP70 family, and protein disulfide isomerase (PDI) are decreased by high

level constitutive heterologous protein secretion¹⁸ and are therefore targets for increasing secretory capacity.

We have investigated the production of McPC603 scFv and several folding mutants as a model system in S. cerevisiae. Tuning the degree of gene amplification through stable δ integration is superior to the use 2µ-based plasmids¹⁷, and can be used to optimize scFv secretion level. Overexpression of BiP or PDI increases secretion titers substantially; and when co-overexpressed, these endoplasmic reticulum (ER) resident folding assistants act synergistically to elevate secretion titers eight-fold. Secretion of up to 20 mg/L active scFv is achieved in shake flask culture by combining expression level tuning and chaperone/foldase overexpression.

Results

Saturation of the secretory pathway of S. cerevisiae. The McPC603 scFv open reading frame was cloned into both centromere-based (CEN) low copy and tunable δ vector integration yeast expression vectors. In the constructs, the scFv is fused to a synthetic pre pro region based on a consensus signal sequence¹⁹ under the control of the inducible GAL 1-10 promoter (δ vector), or a synthetic signal peptide derived from alpha factor under control of the constitutive GAPDH promoter (CEN plasmid). Wild-type McPC603 scFv and three mutant scFvs, which yield higher functional Fv levels in the periplasm of E. coli¹¹, were expressed in yeast. The mutations are located in the heavy chain variable region, and the proteins in increasing order of Fv production are double mutant H3 (S63A, A64D), single mutant H1 (P40A), and a combination of these two sets of mutations, H11 (P40A, S63A, A64D). S. cerevisiae secretes these scFvs in the same rank ordering (CEN plasmids, data not shown) as that observed for the Fvs in E. coli¹¹, with the H11 mutant producing about seven-fold greater scFv titers than the wild-type (Table 1). Expression at 20°C increases scFv secretion by three-fold for the H11 mutant (Table 1).

The scFv expression cassettes were chromosomally integrated using the δ vector. The δ integration vector carries the bacterial NEO gene and results in stable integration of multiple gene copies into the

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Ty δ sequences of *S. cerevisiae* chromosomal DNA¹⁶. In general, the NEO conferred geneticin resistance of a clone increases as the integrated copy number increases, thus allowing the tuning of gene copy number by varying the geneticin resistance used for clone selection. Using this methodology, the optimal number of gene copies encoding for the secretion of a particular protein can be identified¹⁷. Transformants were selected on plates supplemented with 100, 500, or 1000 µg/ml geneticin. Ten colonies were selected from the plates at random and screened by Western blot for maximum scFv secretion. Optimization of expression of each of the scFv constructs using the integrating δ vector resulted in approximately six- to ten-fold increases in protein production over low copy plasmid levels (Table 1).

To further elevate production, an optimally tuned δ strain of wild-type McPC603 scFv was transformed with an integrating plasmid for overexpression of Gal4p, a UAS transcriptional activator for the GAL1-10 promoter. Gal4p overexpression increases transcription from the GAL 1-10 promoter²⁰. Upon induction with galactose, however, the secretion levels for the Gal4p transformed δ strain decrease (Fig. 1A and B), and these strains exhibit a high frequency of enlarged cells compared to the wild-type δ strain control (data not shown). Whole cell protein extracts and Western blot analysis indicate that the scFv in the Gal4p overexpressing δ strain accumulates in an ER-processed pre-Golgi form, pro-scFv, retaining the pro region that is normally cleaved by the Golgi-resident Kex2 protease to yield mature scFv (Fig 1A). Similar secretory "crashes" were observed when Gal4p was overexpressed in other δ transformant strains varying in wild-type McPC603 scFv gene dosage, indicating that elevated Gal4p increases expression levels far beyond the optimum for this protein. Reduction in secretion upon oversaturation of the pathway has also been seen for BPTI¹⁷. Reversal of secretory oversaturation by BiP and/or PDI overex**pression.** When BiP is overexpressed tenfold²¹ in a δ strain expressing wild-type McPC603 scFv, the secretion titer increases 2.4-fold. Similarly, overexpression of rat PDI increases titers 2.3-fold (Fig. 1B). Co-overexpression of BiP and PDI increases wild-type scFv secretion titers eight-fold (Fig. 1B). Overexpression of BiP or PDI eliminates the Gal4p induced crash of the secretory pathway resulting in secretion levels equivalent to non-Gal4p overexpressing strains (Fig. 1B). In conjunction with overexpression of Gal4p, cell density in liquid culture was decreased by 30% whereas there is only a 10% decreased in cell concentration in Gal4p overexpressing δ strains that co-overexpress BiP and PDI. This decrease also corresponded to a decrease in the number of enlarged cells. To test the maximal secretory capacity of scFv in yeast, an optimized δ strain expressing scFv mutant H11, and overexpressing both BiP and PDI, was grown to OD_{600} =60 in shake flasks. This strain produced 20 mg/L of active scFv. By manipulating the expression temperature, vector system, and BiP and PDI levels, an approximately 50-fold increase in scFv specific productivity was achieved (Table 1). This productivity is comparable to that of McPC603 scFv and Fab fragments expressed in E. coli (up to 1.7 mg/L/OD₅₅₀)^{11,22}. A precise comparison is not possible given the many differences between these

expression systems and the antibody sequence also adds to variability in production. *S. cerevisiae* produced scFv was greater than 98% active for all McPC603 constructs, as determined by hapten affinity chromatography and Western blot analysis. Nearly all scFv bound and specifically eluted from the column. Co-overexpression of BiP and PDI did not affect the percentage of active H11 scFv obtained in comparison to nonoverexpressing strains. Purified H11 McPC603 scFv had an equilibrium binding association constant (K_a) of $1.0\pm0.1\times10^5$ M⁻¹ consistent with that of *E. coli* produced material (K_a= 1.3×10^5 M⁻¹ for wild-type McPC603 scFv)²³.

BiP and PDI effects on secretion of several scFvs. Because the δ integration system can lead to increased scFv concentrations in the ER and overload the processing machinery upon Gal4p overexpression, it is of interest to determine if the same effects occur with CEN-based single-copy expression when the scFv concentration in the ER is not as high. BiP overexpression in single copy expression strains leads to 2-5 fold increases in scFv (Fig. 2A). Yeast PDI over-expression also increases scFv yield four- to nine-fold (Fig. 2B).



Table 1. Secretion levels of McPC603 scFvs.

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scFv	Vector	Expression	BiP and PDI	µg/L/OD ₆₀₀
construct		temperature	overexpression	

Figure 1. Effect on the yeast secretory pathway of transcription factor overexpression and BiP and/or PDI overexpression. (A) Western blot analysis of supernatant from McPC603 wild-type scFv δ transformant (pITY-wt, δ vector) compared with intracellular samples of the same transformant with and without Gal4p overexpression (B) Supernatant levels of wild-type scFv and wild-type scFv/Gal4p overexpressing δ strains upon BiP (Kar2p), rat PDI, or BiP and PDI overexpression. Samples were quantitated on a per cell level and normalized to the δ transformant without other overexpressed factors. Exposure time is identical for all samples. Densitometric quantitation was performed for at least five independent transformants and blots to obtain the displayed standard deviations.

H11	δ	20°C	+	330
H11	δ	20°C		120
H11	CEN	20°C	-	21
H11	CEN	30°C		7.0
WT	CEN	30°C	-	1.1

Specific productivity of active scFv is presented for test tube cultures. Scaleup to shake flask results in similar specific productivity and results in 20 mg/L active, purified H11 McPC603 at an $OD_{600} = 60$.





Figure 2. Effects of BiP and PDI overexpression on low copy number (CEN-based) scFv expression. (A) Supernatant levels of wild-type, H1, H3, H11 McPC603 (pRS-GPD, CEN vector), and 4-4-20 (pRS-GAL, CEN vector) upon BiP (Kar2p) overexpression (\Box) versus single-copy production strains (**I**). (B) Supernatant levels upon yeast PDI overexpression (\Box) versus single-copy production strains (**I**). Above the graphs are Western blot data for each construct with and without (A) BiP or(B) PDI overexpression. Secretion was normalized by culture OD₆₀₀, and plotted as the increase relative to that strain without BiP or PDI overexpression. Quantitation was performed for at least four independent transformants. Overexpression of BiP or PDI increased secretion levels with statistical significance *p*<0.01 except for H11 where *p*<0.02. Exposure time of the Western blots varies amongst the 5 scFvs, because basal secretion levels vary 7-fold.



increases are due to chaperone activity or oxidative catalysis. Native rat PDI contains two active CGHC catalytic sites and can act as an oxidase, reductase, isomerase, or chaperone²⁴. When the second cysteine in each of the active sites of the enzyme is changed to serine (CGHS), the oxidase activity is effectively removed while the chaperone activity remains intact and the isomerase activity is slightly diminished^{25,26}. Replacement of the first cysteine in both active sites with a serine residue (SGHC) ablates all catalytic isomerase and oxidase activity and only the chaperone activity remains. The yeast were still viable with SGHC PDI overexpression because the endogenous chromosomal yeast PDI gene remained intact. The same δ transformant expressing wild-type McPC603 (Fig. 1) was transformed with plasmids encoding the rat PDI mutants. Measuring secretion levels with the various overexpressed PDI constructs indicates that the isomerase activity is the primary function of PDI that is required to achieve increased secretion. Overexpressed PDI lacking any catalytic activity (SGHC) had no effect on wild-type scFv production (Fig. 3). Therefore, BiP and PDI increase scFv titers synergistically by affecting protein folding in mechanistically different fashions, leading to the observed synergy in secretory capacity improvement.

Figure 3. Differential effects of mutant PDI overexpression in McPC603 δ production strain (pITY-wt, δ vector). Wild-type δ strains expressed with different PDI plasmids. Lanes 1–2: pRS-314 control plasmid; lanes 3–4: SGHC PDI (pMAL-512) that lacks oxidase and isomerase function; lanes 5–6: CGHS PDI (pMAL-522) that lacks oxidase function; lanes 7–8: wt PDI (pMAL-5.1).

These effects are seen for all four McPC603 scFvs differing in secretion and/or folding behavior (WT, H1, H3, H11) as well as for antifluorescein 4-4-20 scFv which differs appreciably in primary amino acid sequence. The yeast-produced 4-4-20 scFv showed an equilibrium binding dissociation constant, K_d , of $1.3\pm0.3\times10^{-9}$ M, which is comparable to the *E. coli* produced material¹². Thus, it appears that BiP and PDI can increase scFv titers over a range of recombinant scFv protein expression levels, and is generalizable to the several scFvs examined here. **BiP and PDI affect scFv secretion through differing mechanisms.** The expression data do not discriminate between the multiple functions of PDI, and it is not evident whether the observed

Discussion

The effect of varying BiP levels on heterologous secretion is complicated due to BIP's distinct roles in retention of malformed proteins and transient association with normally processed proteins. Substantial evidence indicates an inhibitory role for BiP in secretion of some heterologous proteins such as factor VIII and von Willebrand factor²⁷. Increasing BiP levels, however, increases bovine prochymosin levels in *S. cerevisiae*²⁸ and IgG secretion in *Trichoplusia ni*²⁹ insect cells but not in *Spodoptera frugiperda* insect cells⁴. BiP was first identified in complexes with immunoglobulin heavy chains; light chain BiP binding domains have also been identified³⁰ and there is evidence that the V_H region of an antibody molecule is involved in BiP associated retention³¹. Yeast secretion levels for each of the five scFvs examined in this study are increased upon BiP overexpression, indicating that antibody fragments in general

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may benefit from BiP overexpression. Antibody fragments are particularly prone to aggregation and, depending on the primary sequence and expression conditions, a portion may form insoluble aggregates in the periplasm of *E. coli*^{11,12}. It appears that BiP may solubilize export-competent, native protein in the *S. cerevisiae* ER or bind folding intermediates and thus prevent their degradation. Lowering the expression temperature to 20°C increases scFv secretion three- to five-fold for McPC603 wild-type and secretion mutants in *S. cerevisiae* (Table 1), perhaps preventing aggregation, the same function that is postulated for BiP.

PDI overexpression in S. cerevisiae elevates the production of acid phosphatase, human platelet derived growth factor³², antistasin³³, and a PDI mutant lacking isomerase activity increases human lysozyme secretion³⁴. The isomerase activity of PDI is essential for yeast viability²⁵ and is responsible for the observed increase in scFv titers (Fig. 3). The intrachain disulfide bonds in scFvs are crucial for proper folding and functionality of McPC603, and removal of one or both disulfides leads to unstable proteins in *E. coli*³⁵. In addition, in vitro refolding of reduced, denatured wild-type McPC603 Fv results in the formation of protein aggregates¹¹. Although PDI increases the in vitro refolding yields of an Fab fragment³⁶ and cell-free translation yields of an scFv³⁷, PDI and DsbA overexpression have little or no effect on E. coli antibody production³⁸. In contrast, scFv secretion is elevated by PDI overexpression in S. cerevisiae, and furthermore, co-overexpressing BiP and PDI elevates secretion in a more than additive fashion. The ER environment of chaperones, foldases, and oxidized glutathione is not mimicked in the E. coli periplasm, and the factors that determine maximal yield differ between periplasm and ER. When the level of nascent protein chains present in the ER is increased using δ integration and Gal4p overexpression, the endogenous levels of lumenal folding factors, including any increase resulting from the unfolded protein response³⁹, are no longer sufficient to provide efficient folding and export of scFv. At the very least, BiP and PDI are not present in sufficient amounts, and when overexpressed these molecules are able to recover a secretory pathway saturated by scFv overexpression. BiP may act as an ER detergent preventing aggregation at increased scFv concentration by reversibly binding aggregation-prone folding intermediates; whereas, PDI may break up disulfide aggregates or simply accelerate the folding process, limiting accumulation of scFv folding intermediates in the ER. Because BiP and PDI overexpression do not further increase scFv secretion beyond the maximum obtained prior to Gal4p overexpression, some other process must limit throughput at this level. Nevertheless, their beneficial effect on cell physiology at the high expression level suggests that the amount of misfolded protein is decreased upon their overexpression. Although the mechanistic effects of BiP overexpression on folding intermediates were not elucidated, we have shown that the isomerase activity of PDI is responsible for the effects. Similar production strategies may be successful in handling other disulfide bonded molecules possessing domains with immunoglobulin folds such as the major histocompatibility complex (MHC) and T-cell receptor fragments.

spacer, scFv (V_{H} -(Gly₄Ser)₃- V_{L}), c-*myc* epitope tag, and alpha terminator were excised as EcoRI-BamHI fragments and ligated into the CEN-based yeast shuttle vector, pRS-314 (ref. 41) yielding vectors pRS-GPDwt, H1, H3, H11. A small oligonucleotide linker with restriction enzyme sites EagI-NheI-XbaI-SacI was inserted in pRS-GBPTI⁴⁰ EagI to SacI. The wt scFv gene plus alpha terminator were removed from pUC-scFv construct EagI to XbaI and inserted into pRS-GBPTI. The resulting plasmid, pRS-GALwt, contains the inducible GAL1-10 promoter, synthetic pre pro signal sequence¹⁹ (M-K-V-L-I-V-L-L-A-I-F-A-A-L-P-L-A-L-A-Q-P-V-I-S-T-T-V-G-S-A-A-E-G-S-L-D-K-R), Glu-Ala-Arg-Pro spacer, scFv gene, and alpha terminator. The wt expression cassette was removed from pRS-GALwt by total EagI and partial SacI digests and inserted into the δ vector, pITY-4 (ref. 16) giving pITY-wt. Open reading frames for H1, H3, and H11 were subsequently subcloned into pITYwt as Bsu36I to AflII fragments to yield pITY-H1, H3, H11. The 4-4-20 gene was provided by Dave Kranz (UIUC Biochemistry) and cloned into the pRS-GAL series. The Kar2p overexpressing plasmids pMR-1341 (CEN-URA3) and pGAL-KAR2LEU (CEN-LEU2) result in 10-fold increased galactose inducible Kar2p levels²¹. Overexpression was confirmed under conditions of scFv production by Western blot analysis with anti-BiP primary IgG (data not shown). Rat PDI plasmids (2µ) used are pMAL-5.1, pMAL-512 (C35S/C379S), and pMAL-522(C38S/C382S) and each PDI construct contains the sequence encoding for the yeast ER retention signal, HDEL, rather than the rat KDEL²⁵. Rat PDI overexpression was confirmed under conditions of scFv production by Western blot using anti-human PDI primary antibody (data not shown). The Gal4p overexpression integrating plasmid, pKHINT-C, was provided by L. Schultz at Merck and Co.²⁰ The various plasmids were transformed into Saccharomyces cerevisiae strain BJ5464 (a ura3-52 trp1 leu2 Δ 1 his3 Δ 200 pep4::HIS3 prb1 Δ 1.6R can1 GAL) (Yeast Genetic Stock Center, Berkeley, CA) using electroporation with a BioRad (Hercules, CA) Gene Pulser Transfection Apparatus or lithium acetate transformation⁴². Positive clones were selected using auxotrophic markers for all plasmids except for pITY-wt, H1, H3, H11 for which homologous recombination and integration were selected by geneticin (G418) resistance as described¹⁶. The yeast PDI overexpressing strain, YVH10, is BJ5464 with an additional copy of the PDI gene integrated in tandem with the endogenous copy, and shows a constitutive 16-fold increase in PDI levels³². Production media and protocols. Almost all yeast strains were grown in minimal SD (2% dextrose, 0.67% yeast nitrogen base) medium containing 2XSCAA amino acid supplement (lacking Trp, Leu, and Ura; 190 mg/L Arg, 108 mg/L Met, 52 mg/L Tyr, 290 mg/L Ile, 440 mg/L Lys, 200 mg/L Phe, 1260 mg/L Glu, 400 mg/L Asp, 380 mg/L Val, 220 mg/L Thr, 130 mg/L Gly) buffered at pH 6.6 with 50 mM sodium phosphate with 1 mg/ml BSA as a nonspecific carrier. Wild-type δ strain with pMR-1341 BiP plasmid or pRS-316⁴¹ control used 1% casamino acid supplement (Difco) in place of 2XSCAA because only Ura selection was needed. When necessary, 20 mg/L tryptophan, 200 mg/L leucine, and 20 mg/L uracil were supplemented to the media. Induction medium for all δ strain experiments was identical to growth medium substituting galactose (SG) for dextrose as a carbon source. The δ production strains were grown to saturation in 5 ml SD, centrifuged at 3000×G and the pellet resuspended in an equal volume of SG (2% galactose, 0.67%) yeast nitrogen base) medium for induction and incubated at 30°C for approximately 96 h. Constitutively expressing CEN-based production strains were inoculated into 5 ml SD (SG for BiP overexpressing strains and controls) at an OD₆₀₀ of 0.1 and grown for approximately 96 h at 30°C. The OD₆₀₀ were recorded, the cells harvested at 3000×G, and supernatant samples investigated by Western blot analysis. For maximum production levels, a δ transformant of the well-folding variant, scFv-H11, co-overexpressing BiP and PDI was used. The strain was grown at 30°C in Tunair flasks in SD (2% dextrose, 0.67% yeast nitrogen base, 1% casamino acids) for 72 h to an OD600 of 40, centrifuged at 3000 G, and resuspended in an equal volume of SG (2%) galactose, 0.67% yeast nitrogen base w/o $(NH_4)_2SO_4$, 1% casamino acids) for approximately 48 h at 20°C. After the growth and induction phases, the cells had grown to an OD_{600} of 60. Western blot and scFv quantification. For Western blots, 10-20 µL of supernatant were resolved by SDS-polyacrylamide gel electrophoresis using a 4% stacking and 12.5% resolving gel. The protein was electrophoretically transferred to a nitrocellulose membrane using an Xcell II Novex transfer apparatus (NOVEX, San Diego, CA). The blot was then probed with 9E10 (Babco, Richmond, CA) anti c-myc antibody as 1:1500 dilutions of 9E10 in TBST and followed by an anti-mouse horse radish peroxidase conjugate at 1:2000 dilutions (Sigma, St Louis, MO). Detection of scFv was performed using enhanced chemiluminescence (Amersham, Little Chalfont, UK) and

Experimental protocol

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Strains and plasmids. Wild-type, H1, H3, and H11 McPC603 anti-phosphorylcholine scFv genes were obtained from *E. coli* pLisc_SAF production plas-

mids³⁸. Two oligonucleotides C603-1R and C603-1L (UIUC Genetic Engineering Facility) were inserted into pUC-A-BPTI⁴⁰ EagI-XhoI and sequenced. The oligonuclotides rebuilt the 5' to Bsu36I and AfIII to 3' ends of the scFv and added the nucleotide sequence encoding the 10 amino acid c-*myc* epitope tag, yielding pUC-AOL. The open reading frames of the other scFvs were then subcloned into pUC-AOL using Bsu36I and AfIII sites internally located in the scFv genes. The resulting pUC-scFv cassettes containing the constitutive GAPDH promoter, 19 amino acid alpha factor pre signal sequence (M-R-F-P-S-I-F-T-A-V-L-F-A-A-S-S-A-L-A), Ala-Gly-Arg-Pro

exposure to autoradiography film (Hyperfilm ECL, Amersham). Films were then scanned and quantified using the NIH Image 1.61 program. Band intensities at multiple time points in the linear range of the film were quantitated and the slopes of the intensity versus time plots compared. The exposures shown in the figures are not necessarily from time points quantitated as many of the bands are at saturated intensities. Intracellular protein extracts were performed using TCA precipitation as described³² and samples were prepared by boiling 10 µL TCA protein extract plus 10 µL of 3% SDS-3 mM DTT and analyzed by Western blot. All quantification data were specific per cell production levels normalized by OD₆₀₀. At least duplicate clones at each condition (control and overexpression) were sampled from each of two independent transformations and analyzed. Control strains containing the combination of plasmids pRS-314 (CEN-*TRP1*), pRS-315 (CEN-*LEU2*), and pRS-316 (CEN-*URA3*) that mimic the altered strain were always grown on identical media as the altered strain.

scFv purification. Supernatant was recovered after 2 days of induction at 20°C and applied to a PC-sepharose affinity column, washed with borate buffered saline (4.34 g/L boric acid, 11.4 g/L sodium tetraborate, 29 g/L sodium chloride, pH 8.0), and eluted with borate buffered saline containing 5 mM phosphorylcholine chloride. Percent active scFv was determined by comparing the amount of scFv in the sample and column flow-through by Western blot analysis. Dilutions of the sample were loaded to get direct intensity comparison to flow-through scFv levels. Given that the detection limit of the Western blot protocol is a few picograms, it was determined that, in all cases, greater than 98% of the scFv was active, and often greater than 99%. Affinity measurements. McPC603 H11 scFv: Affinity purified H11 scFv samples were used for the binding affinity determination. At 20°C, phosphorylcholine was added stepwise to 1 µM scFv solutions previously dialyzed extensively against borate buffered saline (4.34 g/L boric acid, 11.4 g/L sodium tetraborate, 29 g/L sodium chloride, pH 8.0). The binding was monitored at the emission wavelength of 328 nm after excitation at 280 nm. Fluorescence measurements were recorded as a function of antigen concentration and fit to an equilibrium binding model to determine the binding association constant, K_a. The scFv protein concentration was determined using A₂₀₅ and an extinction coefficient of $\varepsilon^{1mg/ml}=31$. 4-4-20 scFv: Supernatants from cultures expressing 4-4-20 scFv along with PDI overexpression were extensively dialyzed against phosphate buffered saline (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, pH 7.4). Fluorescein was added stepwise to a 1:6 diluted supernatant sample in PBS at 20°C. Fluorescence was monitored at 510 nm after excitation of the fluorescein dianion at 488 nm. The fluorescence quench resulting from 4-4-20 binding of fluorescein was recorded as a function of fluorescein addition and fit to an equilibrium binding model to determine the binding dissociation constant, K_d.

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