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Phage Selection for Site-Specific Incorporation of Unnatural Amino Acids into Proteins In Vivo

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Abstract—The development of a method for the site-specific incorporation of unnatural amino acids into proteins in vivo would significantly facilitate studies of the cellular function of proteins, as well as make possible the synthesis of proteins with novel structures and activities. Our approach to this problem consists of the generation of amber suppressor tRNA/aminoacyl–tRNA synthetase pairs that are not catalytically competent with all the endogenous *Escherichia coli* tRNAs and aminoacyl–tRNA synthetases, followed by directed evolution of such orthogonal aminoacyl–tRNA synthetases to alter their amino acid specificities. To evolve the desired amino acid specificity, a direct selection for site-specific incorporation of unnatural amino acids into a reporter epitope displayed on the surface of M13 phage has been developed and characterized. Under simulated selection conditions, phage particles displaying aspartate were enriched over 300-fold from a pool of phage displaying asparagine using monoclonal antibodies raised against the aspartate-containing epitope. The direct phage selection offers high specificity for the amino acid of interest, eliminating the potential for contamination with synthetases active towards wild-type amino acids in multiple rounds of selection.

Introduction

In an effort to expand the scope of protein mutagenesis, we previously developed an in vitro method for the site-specific incorporation of unnatural amino acids into proteins.[1] This strategy is based on the suppression of amber stop codons by in vitro transcribed tRNAs that are chemically aminoacylated with unnatural amino acids. To enhance the utility of this technique and provide powerful tools for studying proteins in their native environment,[2] we have been adapting this method to allow the incorporation of unnatural amino acids into proteins directly in living cells.[3–6] The in vivo method relies on delivery of unnatural amino acids to the ribosome by orthogonal suppressor tRNA/aminoacyl-tRNA synthetase pairs.[4–6] In order to aminoacylate the suppressor tRNAs with unnatural amino acids added exogenously to the growth media, the amino acid specificity of each orthogonal aminoacyl–tRNA synthetase (aaRS) must be altered by directed enzyme evolution.

The method for isolation of a mutant aaRS that is specific for an unnatural amino acid from a large pool of aaRS mutants requires a method that is: (i) sensitive since the mutants from the initial rounds could have very low levels of activity; (ii) applicable to a wide range of amino acids, that is a selection that takes advantage of unique chemical reactivity of certain amino acid side chain would be of limited use; (iii) applicable to large libraries—the desired library size for most in vivo selections in *Escherichia coli* is about 10^9 members as determined by the plasmid transformation efficiency or the in vitro phage packaging efficiency; (iv) specific, that is capable of excluding mutants with broader substrate specificities; and (v) tunable, since the ability to control the stringency of the selection from one round to the next may be important. A number of in vitro selection methods can be envisaged including panning of synthetase mutants displayed on phage against immobilized substrates, or a direct high-throughput enzyme assay. Most in vivo selection methods are based on producing a distinct phenotype upon suppression of nonsense, missense or frameshift codons in a reporter gene. For example, in the general antibiotic selection previously reported,[4] the library was passed through a positive selection in the presence of the unnatural amino acid (resulting in a pool of all active synthetases), followed

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by a negative barnase selection in the absence of the unnatural amino acid (yielding only the desired mutants). This selection has been improved by replacing the β-lactamase reporter with the chloramphenicol acetyl transferase (CAT) gene, which has better selection properties due to the bacteriostatic nature of chloramphenicol.[6] However, since the readout of the antibiotic selection is indirect (differences in growth rates conferred by various mutant synthetases, as opposed to the activity of the mutant proteins), the selection results can be significantly skewed by anything that affects growth rates, such as the pharmacology of the unnatural amino acid, reporter gene reversion, or contamination with wild-type reporter or synthetase gene-containing plasmids even at extremely low levels.

Consequently, we developed a more direct selection that takes advantage of monoclonal antibodies specific for an unnatural amino acid presented in the context of a synthetic immunogenic peptide, the poliovirus C3 epitope[7–9] (Fig. 1). A C3 peptide with a TAG stop codon in the middle was fused to the N-terminus of VCSM13 phage coat protein pIII, such that phage production requires suppression of the amber nonsense codon. Cells were then transformed both with a phagemid encoding the synthetase library and the orthogonal tRNA, followed by induction of synthetase expression, and infection with the C3TAG phage. Even a small amount of synthetase activity results in suppression of C3TAG and display of the amino acid substrate on the phage surface. Moreover, each phagemid carrying the synthetase gene is preferentially packaged in the same phage that displays the amino acid, since VCSM13 phage DNA does not have an intact M13 intergenic region necessary for efficient packaging.[10] Subsequently, the phage pool representing all of the active synthetase genes in the library is incubated with immobilized monoclonal antibodies directed against the unnatural amino acid, in order to isolate only the phage carrying the synthetase with the desired amino acid specificity.

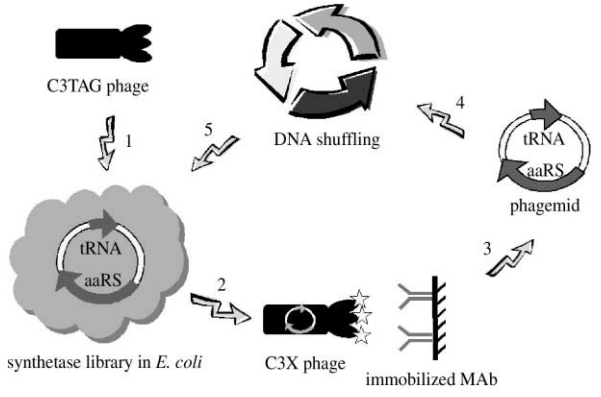

Figure 1. Concept for the phage selection for the incorporation of unnatural amino acids into a displayed epitope. The phage with a stop codon in the displayed fusion peptide gene would be used to infect the selection strain carrying the synthetase library (1); the phagemid DNA coding for active synthetase mutants would be packaged into phage, synthetase substrates would be displayed on the phage surface, and the phage displaying the desired amino acids would be isolated with immobilized antibodies (2); the enriched pool of mutant synthetases (3) could then be subjected to further mutagenesis (4) and passed through additional rounds of selection (5).

One way to analyze the efficiency of selection methods is to dilute the desired molecular species in an excess of undesired species and determine the enrichment for each method after one round of selection. To determine the enrichment in a model phage selection, we took advantage of the suppression properties of two orthogonal systems characterized previously: the glutaminyl[4] and the aspartyl[6] pairs. The glutaminyl orthogonal pair is comprised of the yeast glutaminyl–tRNA synthetase and the yeast $tRNA^{Gln}_{CUA}$; the aspartyl orthogonal pair consists of the yeast aspartyl–tRNA synthetase containing the E188K mutation and the yeast $tRNA^{Asp}_{CUA}$. In a chloramphenicol acetyl transferase (CAT) suppression assay (in which a TAG amber codon at a position D125 in CAT gene is suppressed using the orthogonal pair),[6] the chloramphenicol IC_{50} value for the optimized Gln pair is 350 μg/mL, and for the Asp pair it is 60 μg/mL. The weaker activity of the Asp pair relative to the Gln pair approximates the weak activity of a mutant with novel amino acid specificity in a library containing many more active synthetases specific for the native substrate. Therefore, a substantial dilution of cells containing the Asp pair in an excess of cells containing the Gln pair simulates a library containing a weak hit. The activity of the Asp orthogonal pair can then be used as selectable phenotype in a model selection in order to determine the enrichment properties of the new selection method.

The enrichment value for the phage selection was obtained as follows: the cells containing the weaker aspartyl orthogonal pair were successively diluted into an excess of cells expressing the stronger glutaminyl orthogonal pair, each dilution was infected with the C3TAG-pIII mutant phage, and a selective pressure in favor of the aspartyl pair was applied by affinity enrichment of C3Asp phage over C3Gln phage with anti-C3Asp antibodies. The enrichment value for the phage selection was then determined by comparing the final ratio of Asp/Gln cells to the starting ratio. Here we describe the design of the new phage selection, determine its enrichment properties in a model selection, and discuss its suitability for evolving the amino-acid binding sites of the orthogonal synthetases.

Results and Discussion

Construction of DHRF1 selection strain

To carry out the phage selections, one needs a strain that has high transformation efficiency (to generate large libraries), and that has a deficient release factor 1 (for efficient amber suppression). The efficiency of amber suppression in the release factor 1 temperature-sensitive *E. coli* strain MRA8 at non-permissive temperature (37 °C) is almost an order of magnitude higher than in the wild-type DH10B strain.[6] However, the MRA8 strain is not suitable for selections with large libraries, because its transformation efficiency is typically

only 10^4 cfu/μg plasmid DNA/100 μL electrocompetent cells, or 10^6-fold less than the best cloning strains. Therefore, the *prfA1*^{ts} allele was moved by recombination from MRA8 cells into the DH10B strain to yield DHRF1 strain, which combines high suppression efficiency with high transformation efficiency (see Experimental). The presence of the *prfA1*^{ts} allele in DHRF1 strain was functionally confirmed in a CAT assay (Table 1), in which the suppression with the aspartyl orthogonal pair was compared at permissive (30 °C) and non-permissive (37 °C) temperature. The chloramphenicol IC_{50} values obtained with the new DHRF1 strain at 37 °C were higher than at 30 °C, which confirms the *prfA1*^{ts} phenotype.

Optimization of synthetase expression in DHRF1 strain

The expression levels of the AspRS- and the GlnRS-suppressor tRNA pairs had to be optimized for maximum suppression efficiency in the DHRF1 selection strain. The best system for introducing the aspartyl orthogonal pair involved the use of plasmid pTAK-D. In this construct, synthetase gene expression is under control of the IPTG-inducible *tac* promoter, and the tRNA transcription is under the control of the constitutive *lpp* promoter and the *rrnC* terminator.[6] To make the aspartyl pair suitable for phage selection, the AspRS/suppressor tRNA cassette from pTAK-D was moved to an M13-origin containing phagemid pHD without any observable change in the suppression properties of the pair.

However, GlnRS expression from an analogous $ColE1_{pBR322}$ origin-containing plasmid was toxic to the cells upon induction of the *tac* promoter with 0.1 mM IPTG (pTAK-Q, Table 2). The toxicity was alleviated by replacing the *tac* promoter with the *lac* promoter (plasmids pQD and pQD*rop*$^-$ in Table 2), but the GlnRS expression was toxic even under the *lac* promoter when the orthogonal pair was moved to a higher-copy $ColE1_{pUC18}$ origin-containing phagemid (pBlue-Q in Table 2). To make the glutaminyl pair suitable for phage selection, the M13 origin was added to the pQD(rop-) vector to yield phagemid pHQ which can be packaged by the phage. The performance of the two orthogonal pairs was therefore optimized with the use of different synthetase promoters and a choice of suitable vectors.

Determination of antibody specificity

For the purpose of the model selection experiments, a mouse monoclonal antibody (MAb C3-D) was produced against a synthetic C3Asp peptide (see Experimental for details). We chose the residues 95–104 of poliovirus coat protein VP1 comprising the C3 epitope, PASTTNKDKL, because: (i) it had been found to be very immunogenic,[8] (ii) its crystal structure with a human antibody is available (showing that residue N100 of VP1 is crucial for antibody recognition),[7] and (iii) mutations at the N100 position of VP1 allow poliovirus to avoid immune response.[13] Combined, this evidence suggests that the peptide may either have secondary structure in solution or may form a similar structure upon antibody binding,[14] as do other antigenic peptides,[15] which would contribute to the uniform mode of immunorecognition and explain the high immunogenicity of this sequence. In order to determine the specificity of MAb C3-D for Asp100-containing C3 peptide PASTTDKDKL*GC*, its binding affinity was compared to C3 peptides with various closely related residues at position N100 using ELISA (Table 3). The anti-C3Asp antibody bound C3Asp 25-fold better than C3Glu, and at least 100-fold better than all the other peptides that were examined.

Enrichment in model selections

To demonstrate the specificity of the MAb C3-D against peptides displayed on the phage surface, the C3Asp and

Table 1. CAT suppression test of the *prfA1*^{ts} phenotype of DHRF1 strain

	DH10B		MRA8		DHRF1	
	30 °C	37 °C	30 °C	37 °C	30 °C	37 °C
Active AspRS only	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
Active tRNA + inactive AspRS	<2.0	<2.0	<2.0	3.0	<2.0	3.0
Active tRNA + active AspRS	15	15	15	50	15	50

The IC_{50} values at permissive (30 °C) and non-permissive temperature (37 °C) are in μg/mL chloramphenicol. See Experimental for details.

Table 3. Specificity of MAb C3-D, determined by an ELISA against covalently immobilized C3 peptides

	Amino acid at position X in PASTTXKDKLGC- peptide						
	Asp	Glu	Asn	Gln	Ser	Ser-PO_4[a]	L-AP-4[b]
Dilution	4500	180	40	10	10	25	15

The values are antibody dilutions at which the optical density at 405 nm equals 1.0.
[a]Phosphoserine.
[b]*L*-2-amino-4-phosphonobutyric acid.

Table 2. GlnRS expression in the DHRF1 strain under different promoters and from vectors with different copy numbers per cell, as measured in a CAT suppression assay.

	Origin of plasmid replication		
	$ColE1_{pBR322}$ + *rop*	$ColE1_{pBR322}$	$ColE1_{pUC18}$
tac Promoter	ND[a]	Toxic (pTAK-Q)	Toxic (pSK-Q)
lac Promoter	230 (pQD)	350 (pQD*rop*$^-$ and pHQ)	Toxic (pBlue-Q)

The chloramphenicol IC_{50} values are in μg/ml; vector designations are in parentheses.
[a]ND, not determined.

C3Asn sequences were fused to the T7 phage and the VCSM13 phage coat proteins. There are three phage coat proteins commonly used for peptide display: the 10A protein of the T7 phage, and the pIII and pVIII proteins of the M13 phage. The T7 phage system from Novagen offers high fusion peptide valency—there are 415 copies of C3 peptide fused to the C-terminus of coat protein 10A and displayed on the phage surface. Attempts to fuse the C3 peptide to the N-terminus of the VCSM13 coat protein pVIII, present at 2500 copies per phage, were not successful, probably due to the stringent limits on the length and sequence of the displayed fusion peptides.[16] Therefore, the C3 peptides were fused to the N-terminus of the pIII coat protein, present at five copies per phage. Partial display of fusion coat proteins provided in *trans* to the excess of wild-type coat protein expressed by a helper phage or from a separate vector was avoided, because the epitope valency could depend on the fusion peptide sequence and would be difficult to determine. The Asn100 residue of the C3 epitope was chosen for the competition experiments with the Asp100, since the anti-C3Asp antibody recognized the C3Asn to a measurable degree (Table 3), and the displayed C3Asn peptide can be directly visualized with the MAb C3N, a poliovirus-neutralizing mouse monoclonal antibody (a small aliquot was generously provided by R. Crainic, Institut Pasteur).

For the purpose of measuring enrichments, the only difference between the T7 and the M13 phage systems is epitope density on the phage surface. In each of the three selections in Figure 2, the D phage was serially diluted into an excess of competing phage and enriched with the MAb C3-D. The final fraction of the D phage was then determined either by plaque blotting or by PCR (see Experimental for details). The enrichment value after one round of each selection is the final fraction of D phage divided by the starting fraction. The results demonstrate that the difference of almost two orders of magnitude in epitope density had little effect on the enrichment values obtained with the MAb C3-D: the high epitope-density T7 system afforded a 1100-fold enrichment of D phage compared with 900-fold enrichment obtained with the M13 system. It is notable that the MAb C3-D can discriminate well between two peptides that differ only by one amino acid, despite the avidity effect of the multivalent peptide display.

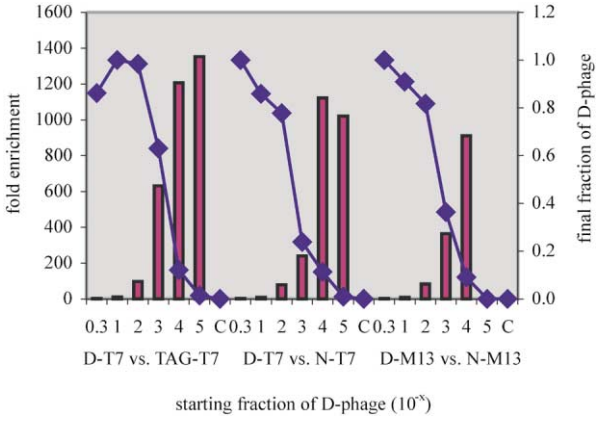

Figure 2. The specificity of the MAb C3-D against peptides displayed on the phage surface. The control set of data on the left represents the enrichment of D-T7 phage over unsuppressed TAG-T7 phage; the two sets of data on the right provide a comparison of the enrichment of D phage over N phage in the T7 and the M13 systems. The left axis corresponds to *columns* which represent enrichment values; the right axis to *diamonds* which are the final fractions of D phage after one round of selection; and *C*s are negative controls without the D phage.

In a real selection applied to a synthetase library, suppression of the stop codon in the displayed epitope would most likely result from the activity of synthetase mutants that still recognize their wild-type amino acid. In fact, the activity of the synthetase mutants with desired unnatural amino acid specificities would probably be relatively low, particularly during the initial rounds of the selection. To simulate such conditions, the enrichment was determined with the newly constructed C3TAG-pIII VCSM13 phage (TAG-M13 phage), which contains a TAG amber stop codon at position 100 in the C3 fusion peptide. The M13 phage is more suitable for the phage selection than the T7 phage for the following reasons: (i) the displayed peptide can be fused to the N-terminus, rather than the C-terminus of the coat protein to prevent phage production in non-suppressing cells harboring inactive synthetase mutants; (ii) the VCSM13 phage is preferentially packaged with phagemid DNA, providing a convenient linkage between the displayed synthetase substrates and the corresponding synthetase genes; and (iii) the M13 phage titers are two orders of magnitude greater than those of the T7 phage. To test the new construct, the N-terminal C3TAG fused to the pIII protein was suppressed with each orthogonal pair. The less active aspartyl pair produced less phage during the 12-h suppression period than the more active glutaminyl pair. The difference between the suppression efficiencies of the two orthogonal pairs mimics the real selection conditions, where the desired weak synthetase activity (i.e., the AspRS) must be isolated from an excess of undesired strong synthetase activity (i.e., the GlnRS). Therefore, serial dilutions of cells carrying the AspRS pair in an excess of cells expressing the GlnRS pair were infected with the TAG-M13 selection phage, the phage displaying aspartic acid in the C3 epitope was isolated with MAb C3-D immobilized on a micro well plate, and the final ratio of AspRS phagemid to GlnRS phagemid was measured to determine the enrichment. The observed enrichment value of 800 is comparable to the enrichment value of 900 obtained in the VCSM13 selection without suppression in the C3 fusion peptide in Figure 2. The decrease in enrichment could be attributed to a distortion of the initial D phage fraction, caused by the faster Q phage production by the more efficient Gln orthogonal pair.

To further increase the stringency of the model selection, the enrichment of the Asp pair was measured against the *supE* suppressor tRNA, which is a more efficient amber suppressor than the Gln orthogonal pair (*supE* codes for an *E. coli* suppressor $tRNA^{Gln}_{CUA}$, which is efficiently aminoacylated by the endogenous *E. coli* GlnRS). In a β-lactamase suppression assay, the plasmid

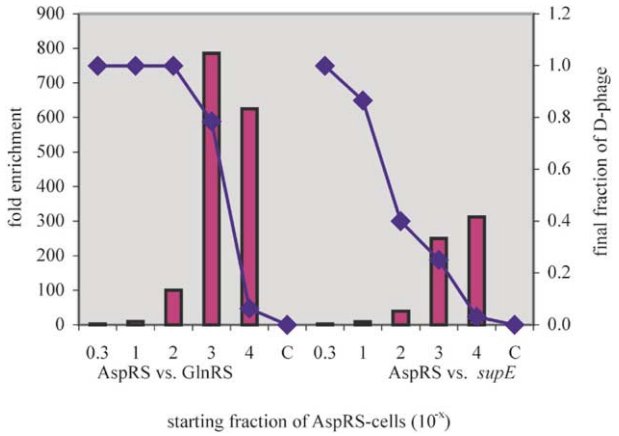

Figure 3. Model phage selection with the TAG-M13 phage. See Figure 2 for legend. Cells carrying the AspRS pair diluted in an excess of cells expressing the GlnRS pair or the suppressor tRNA *supE* were infected with the TAG-M13 selection phage, the phage displaying aspartic acid in the C3 epitope was isolated with immobilized MAb C3-D, and the final fraction of D phage was measured to determine the enrichment.

pAC-*supE* confers ampicillin resistance up to the IC_{50} of 2000 μg/mL, 4-fold higher than the Gln orthogonal pair.[4] In the more stringent model phage selection, the enrichment value decreased to 300, less than 3-fold from the previous experiment, which is also attributable to the faster Q phage production by the more efficient *supE* suppressor. Although both the *supE* and the Gln orthogonal pair suppress the C3TAG more efficiently than the AspRS pair, the D phage was still efficiently recovered from the final phage pool. It is reasonable to assume that 1 in 10,000 or 1 in 100,000 synthetase mutants in a typical library are active towards the desired substrate; therefore the enrichment value of 300 obtained for a starting 10,000-fold dilution of AspRS cells in our model selection in Figure 3 is representative of the enrichment we would expect to observe under real selection conditions. These results suggest that the selection based on suppression of the VCSM13 phage coat protein is a powerful method for isolating active aminoacyl–tRNA synthetase mutants with novel amino acid specificities.

Conclusion

The specificity of monoclonal antibodies combined with the ability to link the product of the aminoacylation reaction with the enzyme-encoding DNA through phage affords a powerful selection for evolving the amino-acid binding sites of aminoacyl–tRNA synthetases. A high enrichment of up to 300-fold per round of selection was observed under model conditions that approximate a real selection, which validated the utility of this novel method. The phage selection will now be applied various aminoacyl–tRNA libraries tailored to a number of unnatural amino acids in an effort to expand the genetic code and to develop an in vivo method for the site-specific incorporation of unnatural amino acids into proteins.

Experimental

Strains, plasmids and reagents

Strains DH10B, XLBlue, and MRA8 were obtained from GIBCO/BRL (Carlsbad, CA), Stratagene (La Jolla, CA), and J. Barrett (University of California, Berkeley, CA, USA), respectively. Homologous recombination plasmids pJW168 and pLoxGen4 were obtained from F. Bolivar (Universidad Nacional Autonoma de Mexico, Mexico),[12] and MAb C3N from R. Crainic (Institut Pasteur, France). All F′-episome bearing strains were obtained by F′ transfer from XLBlue strain by bacterial mating.[11] The *Taq* DNA polymerase was purchased from Stratagene, shrimp alkaline phosphatase and dNTPs from Roche Molecular Biochemicals (Nutley, NJ), Klenow fragment and restriction endonucleases from New England Biolabs (Beverly, MA), synthetic oligonucleotides from Operon (Alameda, CA) and Sigma Genosys (Woodlands, TX), and the Fmoc-protected amino acids for peptide synthesis from Novabiochem (Darmstadt, Germany).

Construction of the DHRF1 strain

Genomic DNA was extracted from the *prfA1*^{ts} strain MRA8 by standard methods.[17] The *prfA1*^{ts} gene and 1 kb of the adjoining upstream sequence were isolated by PCR with primers 5′-GGCTGACTAGTGCCAGC-GGCCTGGATTCACTGG-3′ and 5′-GGCTGC TCGAGTTATTCCTGCTCGGACAACGCCGCC - 3′, and subcloned between the *Spe* I and *Xho* I sites in pLoxGen4 to afford pLoxGen-A. The 1 kb sequence downstream from *prfA1* was amplified with primers 5′-GGCTGCATATGTGGAATATCAACACTGGTTAC-GTC - 3′ and 5′ - GGCTGGCATGCGCCATCAACG-CAATGCCGCTTAAC - 3′; and subcloned between the *Nde* I and *Sph* I sites in pLoxGen-A to yield pLoxGen-AB. To facilitate recombination in the *recA* deficient strain DH10B, the *recA* gene was isolated from the same genomic DNA by PCR with primers 5′ - GGC-TGTCTAGAGCCACTGC CCGCGGTGAAGGC - 3′ and 5′ - GGCTGAAGCTTGCGACCCTTGTGTAT-CAAACA AGACG-3′; and subcloned between the *Xba* I and *Hind* III sites in the plasmid pJW168 to afford pRecA. The plasmid pRecA does not propagate at 37 °C, because it has a temperature - sensitive replicon derived from plasmid pSC101.[12] The recombination cassette fragment containing *prfA1*^{ts} and its neighboring sequences, interrupted by the gentamycin resistance marker flanked by two *loxP* sites, was excised from *Hind* III and *Nco* I sites and inserted between the same sites in pRecA to generate pRecA-LoxGen-prfA1. After removal of the ampicillin resistance marker between the *Bsa* I and *Sca* I sites, this temperature-sensitive plasmid was grown in DH10B cells for 12 h at a permissive temperature (30 °C) in the presence of gentamycin to allow for plasmid integration; the culture was then plated at a non - permissive temperature (37 °C) on gentamycin plates to eliminate the plasmid, followed by screening of individual colonies for the *prfA1*^{ts} phenotype by comparing growth at 42 and 37 °C. The candidate strains were tested in a CAT suppression assay by co-transforming the cells with the CAT reporter plasmid

pACM(D112TAG),[6] and the AspRS orthogonal pair vector (pTAK - DRS^{E188K} - YD_{CUA}) or one of the corresponding control plasmids (pTAK - $DRS^{E188K,A5}$ - YD_{CUA}, and pTAK - DRS^{E188K}). The chloramphenicol IC_{50} values were determined as described previously.[6] The transformation efficiency of the new DHRF1 strain is similar to that of DH10B.

Construction of the orthogonal pair expression vectors

The aspartyl pair vector pTAK-D is identical to the pTAK-DRS^{E188K}-YD_{CUA} plasmid, and the plasmid pTAK-Q is the yeast glutaminyl pair analogue of pTAK-D. The pHD phagemid was constructed by inserting the *EcoR* V/*Nsp* I aspartyl pair fragment from pTAK-D between the *Sph* I and *BstZ17* I sites in pSA01151. The plasmid pQD*rop*$^-$ is similar to pTAK-Q, except that the synthetase is behind a *lac* promoter instead of the *tac* promoter; pQD*rop*$^-$ was made by excision of the *rop* gene between the *BstZ17* I and *Msc* I sites in the pQD vector. The pHQ phagemid was made by inserting the *Pvu* I/*AlwN* I fragment with the M13 origin from the pMAL-p2x plasmid (New England Biolabs) between the identical sites in the pQD*rop*$^-$ vector. Insertion the *tac*-GlnRS fragment isolated from pTAK-Q by PCR between the *Sap* I and *AlwN* I sites in pBluescriptSK$^-$ (Stratagene) afforded the plasmid pSK-Q. Replacement of the *Sap* I/*Avr* II *tac* promoter fragment in pSK-Q with an analogous *lac* promoter fragment from pQD*rop*$^-$ yielded plasmid pBlue-Q. The CAT suppression assay was performed as above.

Production of the anti-C3Asp antibodies

The C3 peptides PASTTXKDKL*GC,* where X = Asp, Glu, Asn, Gln, Ser, Ser-PO_4, and *L*-2-amino-4-phosphonobutyric acid were synthesized using standard Fmoc chemistry on a 0.1 mM scale with the Model 432A Applied Biosystems (Foster City, CA) peptide synthesizer, deprotected and cleaved off the resin with Reagent K,[18] purified by HPLC, and analyzed by MALDI-MS. The C3Asp peptide was then conjugated to the maleimide-activated KLH (Pierce; Iselin, NJ) via the C-terminal cysteine according to manufacturer's instructions, and the conjugate was used to immunize mice. Mouse monoclonal antibodies were generated by standard hybridoma technology.[19] The specificity of the antibody was determined by ELISA, in which serial three-fold dilutions of the antibody stock (25 mg/mL) were added to the peptides immobilized via cysteines on Reacti-Bind maleimide-activated 96-well plates (Pierce). The binding to each peptide was quantified with a secondary anti-mouse antibody conjugated to alkaline phosphatase. For each peptide, the optical density of the hydrolyzed *p*-nitrophenyl phosphate substrate at 405 nm was plotted as a function of antibody dilution, and the dilutions at an arbitrarily chosen $OD_{405} = 1.0$ were summarized in Table 3. We attempted to generate rabbit polyclonal antibodies as well, but as one might expect, they were not nearly as specific as the mouse monoclonal antibodies.

Construction of the phage fusions

To generate the C3-T7 phage fusions, synthetic complementary 65-mer oligonucleotides coding for the C-terminal GGPASTTXKDKLG peptide (where X is coded as Asp, Asn or amber codon) were denatured at 90 °C for 5 min, annealed by slow cooling to 25 °C, digested with *EcoR* I and *Hind* III enzymes and purified on 3% agarose gels. The short restriction fragments were then ligated with two linear T7 DNA fragments predigested with the same enzymes by the manufacturer, and packaged in vitro using the T7Select Cloning Kit (Novagen; Madison, WI). Similar C3 sequences coding for the N-terminal PASTTXKDKL peptides were inserted between the leader and the mature gIII sequences in the VCSM13 interference-resistant phage (Stratagene) by Kunkel mutagenesis using 75-mer reverse primers. The phage DNA from the fusion constructs was sequenced and the peptide display was confirmed by dot blot analysis using the anti-C3Asn and anti-C3Asp monoclonal antibodies. The TAG-M13 phage was cloned and propagated in the MRA8 cells deficient in release factor 1, which has a strong suppressor phenotype conferred by the pAC-*supE* plasmid.

Phage selections

In the D/N-T7 selections the desired dilutions of CsCl-purified phages were mixed, then each mix (10^{10} pfu/mL) was added at 100 μL per well into three wells in a MaxiSorp 96-well plate (Nalgene; Rochester, NY) coated with 10 μg/mL of the MAb C3-D, incubated for 2 h, and washed three times with PBS + 0.1% Tween and three times with PBS using the EL-504 Magna automated plate washer (Bio-Tek). The bound phage was recovered by adding a fresh culture of host cells, combining the three wells, and plating serial dilutions of the infected culture to obtain plates with at least 100 dispersed plaques. The phage ratio after the antibody selection was determined by plaque blot analysis with the MAb C3-D and C3N antibodies. The D/N-M13 selections were performed in the same manner, except that the readout was performed by PCR on approximately 20 individual plaques per plate, because the M13 plaque blots with the anti-C3 antibodies showed only weak signals due to the lower display rate of C3 peptides per phage.

In the suppression selections, 5 mL cultures of DHRF1 F' cells containing either the pHD or the pHQ phagemid bearing the corresponding orthogonal pair were grown to $OD_{600} = 0.8$, the synthetase expression was induced with 0.1 mM IPTG for 1 h, each culture was then infected with 5.0×10^{10} pfu of TAG-M13 phage for 30 min at 37 °C, and washed four times with PBS. The dilutions of cells were mixed in various ratios and grown at 37 °C in 5 mL cultures for 12 h in the presence of ampicillin, tetracycline and IPTG. The cells were removed by centrifugation, and the selection was performed with the supernatant as before. The final fractions of D phage were obtained by PCR analysis of 20 individual plaques for each initial dilution of C3Asp cells.

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