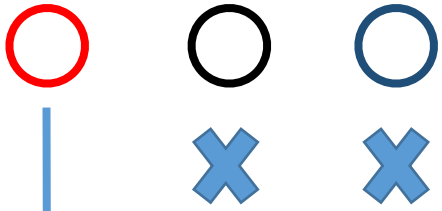
Addicting diverse bacteria to a non-canonical amino acid

TEM-1 B-Lactamase

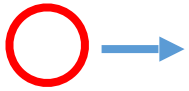
Aim to make functional TEM-1 dependent on presence of non naturally occurring NCAA (3nY/3iY) ***AND*** minimize reversion away from NCAA

L162 F66 T189



3 **candidate positions** found where incorporation of NCAA disrupts function
OTS incorporates NCAA at Amber stop codon

Library of variants of the amino acids spatially close to candidate positions, recover variants with activity dependent on NCAA.



→ Reversion to tyrosine (UAG to UAC/UAU) → Force tyrosine incorporation at position 162 and select variants that lose function → 23 variants

23 variants → Reversion to leucine (UAG to UUG) → 4 variants → Reversion to any other amino acid → 1 variant, TEM1.B9, was found to only retain activity when amino acid 162 reverted to phenylalanine

TEM1.B9 → 250 generations, no escape events

They identified three positions (L162, F66, T189) where substituting for a non canonical non naturally occurring amino acid would disrupt the function of the antibiotic resistance protein TEM1 protein. They mutate at the DNA level so that instead of the WT codons, the protein coding sequence reads for the amber stop codon (rare stop codon) UAG at the stated positions. They express the three protein mutants in E.coli with an orthogonal translational system (basically tRNA that incorporates the NCAA at amber stop codons, thereby creating mutant proteins with NCAA at positions stated. Observe that as expected protein function is abolished. Then randomly vary the 6 amino acids closest to each of these positions to create three libraries of variants with the hope some of these variants will restore function. Identify functional protein, however in libraries of F66 and T189 variants restoration of function is not due to variation introduced but rather because the amber stop codon has mutated back to the WT at the DNA level. So these libraries are scrapped. The L162 library however contained >95% proteins that retained the amber stop codon and also restored protein function due to the variation introduced. Now they have what they were looking for, functional protein with NCAA in a critical functional position. However the second challenge is to make sure that the amber stop codon cannot be lost without losing function as well. Loosing the amber stop codon through mutation is referred to as an escape event. The two most likely escape event to occur are to a codon encoding for tyrosine or leucine as these require only a single point mutation from the stop codon. So they decide to force the introduction of tyrosine into the 162 position by using an alternative OTS that introduces tyrosine at the amber stop codon. They do this and rule out any proteins that retain their function when there is tyrosine at position 162. They are now down to only 23 variants. They then force incorporation of leucine to the 162 site to each of the 23 variants, this time instead of using a third OTS they simply mutate the protein coding sequence of the DNA to encode leucine rather than the stop codon. Again they discard proteins that are still functional with leucine at position 1632. They now have only 4 variants remains. No other escape events can occur by only a single point mutation, but for completeness they mutate the coding seq of each of the variants to incorporate each of all of the amino acids at position 162. They look for functional proteins. Various of amino acids can be incorporated at position 162 without disruption function, particularly phenyl alanine. Variant TEM1 B.9 is only functional with phenylalanine and no other amino acid at 162 (except of course our NCAA). This is therefore the best variant. The authors reason that seeing as phenylalanine cannot be reached by a single point mutation the they don't have to worry about escape to this amino acid. They passage E.coli expressing this variant of the antibiotic protein in the presence of the antibiotic and NCAA and appropriate OTS for 250 generations and detect no escape events. Resistance is dependent on presence of NCAA. They say the protein is "addicted" to the NCAA. They subsequently express the system show same results in various other organisms