Chemical chaperones assist folding through different mechanisms to buffer mutational variations

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Chemicals and Reagants

All the chemicals used in this study were from commercial sources and were of analytical grade. Media components, fine chemicals and reagants were purchased from Sigma Aldrich (St Louis), HiMedia (Mumbai, India). Oligonucleotides were purchased from Sigma Aldrich. KAPA HiFi HotStart ReadyMix obtained from KAPA Biosystems. Gel extraction kit obtained from Qiagen (Valencia, CA), AntiMBP polyclonal antisera obtained from New England Biolabs (Beverly, MA) and Goat Anti-Rabbit horseradish peroxidase-linked antibody were bought from GeneScript. Immobilon PVDF membrane and chemiluminescence reagent for western blotting purchased from Millipore (Billerica, MA). Antibodies against DnaK, and GroEL were obtained from Assay Design and anti-TF antibody was obtained from Genscript. ΔdnaK and ΔotsA deletion strains were obtained from the E. coli genetic stock centre at Yale University.

Site Specific Mutagenesis of Gentamicin acetyl-transferase gene (Gm-R): Primers were designed to replace each of the two adjacent amino acid residues with glycine and were obtained as reverse primers in 96-well format (Sigma). Pair of amino acid residues starting from position 5th till 172th were replaced by two Glycine residues using mega primer based method of site-directed mutagenesis. First reaction was carried out in 96-well PCR plate, with each well having specific mutagenic primer and a forward template-specific primer. Twenty five cycles of PCR were carried out at annealing temperature of 55^{0}C. Short product obtained, served as mega-primer to obtain full-length mutant plasmid. Thirty cycles of long PCR were carried out with both annealing and extension steps performed at 72°C. WT template (Gentamicin Acetyl Transferase WT cloned into pBAD vector), was digested with DpnI followed by transformation into chemically competent *E.coli* (DH5α) from Invitrogen.

Screening for the effect of osmolytes on folding of Gm-R Double Glycine Mutants: Gentamicin double glycine variants transformed into *E.coli* strain DH5α were plated onto LB-agar plates containing Ampicillin (100μg/ml), in small spots of 10μl. Mutant colonies were picked and inoculated in LB-Broth containing Ampicillin(100μg/ml) in 96-well plate and were grown overnight at 37^0C. For checking effect of TMAO on growth of variants, secondary culture was inoculated in two parallel sets, control and test plate. Control plate contained Ampicillin(100μg/ml) and Gentamicin (7μg/ml) for selection, and Arabinose (0.1%) for inducing expression of gentamicin acetyl-transferase variants. Test plate contained TMAO (200mM)/proline(200mM)/glycerol(10%) in addition to the constituents present in control plate. In both the sets wild-type was inoculated in wells H11 and G11. Secondary cultures were grown overnight without shaking at 37^0C. Growth of Wild-type and Variants, in presence and absence of TMAO, proline and glycerol, were checked by measuring absorbance at 600nm in an ELISA plate reader. Absorbance of the blank was subtracted and absorbance value for each mutant was normalised w.r.t Gm-R wt grown under the same media condition.

Solubility of Gm-R Wt and Glycine duplet mutant N135G,A136G : Both Gm-R Wt and GG mutant were grown in LB(control) and LB containing TMAO(200mM) with and without induction of protein expression. Cultures were grown till O.D 600nm reaches 0.6 and induced with 0.5% arabinose. Both induced and un-induced samples were allowed to grow overnight at 37^0C. cells were harvested next day and resuspended in 100μl of Baclysis solution (GenScript)containing 1KU/ml of lysozyme(Sigma). Following resuspension samples were incubated at room temperature with constant mixing for 30min. Cells were lysed in three

cycles of freeze thaw. To separate out supernatant and pellet fractions cell lysate was centrifuged at 13,000g for 20 min. Supernatant was collected in separate tubes and pellet was resuspended in 100µl of PBS buffer. Protein concentration of the supernatant fractions were estimated using Bradford estimation method and equal amount of protein was loaded onto 12-15% polyacrylamide gel. For pellet fractions volume corresponding to respective soluble fraction was loaded. Gels were stained with coomassie brilliant blue and the image was scanned and analysed in ImageJ software [1] (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011.)

Targeted-Random Mutagenesis of Gm-R : Library of Targeted-Random mutants was generated by replacing each amino acid of the Gm-R sequence by nineteen other amino acids. Reverse primers were designed such that each codon is replaced with NNK (N= any nucleotide, K= G or T), these were obtained in 96-well format(Sigma). First reaction was carried out in 96-well PCR plate, with each well having specific mutagenic primer and a forward template-specific primer. Twenty five cycles of PCR were carried out at annealing temperature of 55^{0}C. Short product obtained, served as mega-primer to obtain full-length mutant plasmid. Thirty cycles of long PCR were carried out with both annealing and extension steps performed at 72^{0}C. WT template (Gentamicin Acetyl Transferase WT cloned into pBAD vector), was digested with DpnI followed by transformation into chemically competent *E.coli*(DH5α) . Around fifty colonies were picked for each position and pooled for plasmid preparation. This pool of plasmids carrying mutant templates was transformed into E.coli-K12 BW25113 strain and plated onto LB-agar plates containing Ampicillin (100µg/ml). This plate was used as master plate for Replica plating onto control plate containing Ampicillin and Arabinose(0.5%) and test plate containing ampicillin, arabinose

and gentamicin (4µg/ml). 762 Inactive colonies were picked into LB-Broth containing Ampicillin(100µg/ml) in 96-deep well plates. Out of 762 inactive colonies 20 were sequenced and single site mutations were confirmed.

Screening for the effect of osmolytes on folding of Gentamicin Random Mutants:

Out of 762 inactive colonies, 500 were randomly picked for initial screening. These inactive mutants were grown in LB(control) and LB containing osmolytes, [TMAO(200mM)/ Proline(200mM)/ Glycerol(10%)/ NaCl(0.75M)] at 37^0C without shaking. Both the test and control plates contained Ampicillin(100µg/ml), Arabinose(0.1%) and Gentamicin (8µg/ml).

Minimum Inhibitory Concentration(MIC) study of Gm-R targeted random mutants : from screening experiment around 90 mutants that were found to be dependent on osmolytes for growth were picked up for MIC study. These mutants were grown overnight without shaking in LB containing Ampicillin(100µg/ml) and Arabinose(0.1%) at 37^0C. Secondary inoculations were done in LB(control) and LB containing three different osmolytes: TMAO(200mM)/ Proline(200mM)/ Glycerol(10%) and six different gentamicin concentrations (0.5, 4, 8, 16, 32, 64 µg/ml). Growth in each condition was assessed by measuring absorbance at 600nm. Absorbance value for each of the mutant under all conditions was normalised w.r.t. its Sub-MIC Abs value

Western Blotting Experiment : E. Coli K-12 BW25113 was inoculated in plain LB and LB containing TMAO(200mM)/ Proline(200mM)/ Glycerol(10%) and was grown at 37^0C for three hours. O.D 600nm was determined following which cells were harvested and resuspended in PBS buffer leading to 1:150 dilution of the absorbance value. Equal volume

of samples were loaded onto 12.5% poly-acrylamide gel. Proteins were transferred onto the PVDF membrane and were probed using DnaK, GroEL specific monoclonal antibody (assaydesigns) isolated from mouse and TF-tag(GeneScript), DnaJ(assaydesigns) specific polyclonal antibody isolated from Rabbit. All steps were performed on a SNAP-ID system from Millipore. Bands corresponding to DnaK and TF were probed on the same blot and GroEL and DnaJ were probed on another blot. Antibodies were stripped off by incubating in stripping buffer (62.5mM Tris,2%SDS,100Mm β-ME, pH6.7) at 55^0C for 10min.Blot was developed using HRPconjugated Goat anti-mouse and anti-Rabbit IgG (Genscript) and H_2O_2 and luminol as substrates(Millipore).

Microarray experiment : E. Coli K-12 BW25113 was inoculated in plain LB and LB containing TMAO(200mM)/ Proline(200mM)/ Glycerol(10%) and was allowed to grow overnight at 37^0C. Cells were harvested and resuspended in PBS buffer(100µl) and RNA later (500µl). Microarray experiments were performed by Genotypic Technology Pvt. Ltd., India. Briefly, total RNA was extracted by using Qiagen's RNeasy minikit. Polyadenylation and Precipitation (PAP) labeling was done followed by T7 promoter based-linear amplification to generate labeled complementary RNA. Hybridization was done onto Agilent gene expression E.coli 8X15k chip, using Agilent's In situ Hybridzation kit.

Microarray data analysis : Data analysis has been done using GeneSpring GX version 11.5 and Microsoft Excel. Normalisation method employed is Percentile Shift Normalization. List of genes that are differentially regulated upon heat and osmotic shock, including the fold change in their expression under these conditions was obtained from Thusitha S. Gunasekera

et al paper [2] available at http://www.med.wright.edu/bmb/op/papers/osmo_Temp/. Fold change in the expression of these listed genes, in the presence of different osmolytes w.r.t their expression in LB, was compared with the available data and plotted in the heat map using Genesis software [3].

MIC experiment for ΔDnaK and ΔOtsA strain : Gm-R Wt and GG mutants were transformed in E.coli K-12 deletion strains and were grown in LB(control) and LB containing 200mM TMAO in presence of different concentration of Gentamicin. Cultures were grown overnight at 37^0C and the Absorbance at 600nm was measured. Absorbance value of each of the mutant under all conditions was normalised w.r.t. O.D 600 value for growth at sub-mic concentration of gentamicin.

Screening for the effect of osmolytes on the activity of CcdB Mutants: CcdB mutant plasmids were obtained as a kind gift from Prof. Raghavan Varadarajan (*Molecular Biophysics Unit, Indian Institute of Science, Banglore 560 012)* in 96-well format. These mutant plasmids were transformed in *E. coli* K-12 BW25113, transformants were grown in LB-Broth containing Ampicillin(100µg/ml) and 0.5% glucose to repress the expression of CcdB variants and were grown overnight at 37^0C. For analysing the effect of osmolytes on activity, secondary culture was inoculated in two parallel sets, control and the test plate. Control plate contained Ampicillin(100µg/ml) for selection and Arabinose(0.001%) for inducing expression of the Ccdb mutants. Test plate contained TMAO (200mM)/ Proline(500Mm)/ 10% Glycerol in addition to the constituents present in control plate. Secondary cultures were grown overnight without shaking at 37^0C. Growth in control and test plates was analysed by measuring absorbance at 600nm in ELISA plate

reader. Absorbance of the blank was subtracted from each well to calculate the growth.

Unlike Gm-R, increase in Ccdb activity inhibits cell growth hence the bc

was calculated as follows.

Activity(A) = (Highest Absorbance Value (corresponding to Inactive mutant)-Absorbance value for each mutant)/Highest Absorbance Value

Typically the highest absorbance value is obtained for an untransformed cell, or cells transformed with an inactive mutant of CcdB.

Cloning of DM-MBP, SM-MBP, oxDM-MBP (18, 296) for integration in yeast.

The DM-MBP, SM-MBP, oxDM-MBP (18,296) in pCH plasmid were PCR amplified using oligonucleotides MBPNheI_FP and MBPAvrII_RP. The 1.3-kilobase PCR product obtained was digested with NheI and AvrII and cloned into single copy, pYM-N23 vector [4]downstream of the *GAL1* promoter and with a HDEL ER retention signal at C-terminus.

Yeast strains, media and growth conditions

The list of yeast strains used in this study is shown in Table S2.

S.cerevisiae was routinely maintained on YPD medium. The YP medium for Galactose induction contained Yeast extract and peptone together with 2% Raffinose & 2% Galactose as carbon source.

Yeast DNA isolation and yeast transformation

Yeast chromosomal DNA was isolated using Real Biotech Cooperation Genomic DNA isolation columns together with Sorbitol buffer and Zymolase. The yeast transformation procedure used was as described by[5] .

Construction of Strains

The genotypes of strains used in all the experiments are listed in Table I and their construction is described below.

The *S.cerevisiae* strain YMJ003 bearing UPRE-GFP-*TEF2pr*-RFP-*MET15-URA3* cassette integrated at *URA3* locus in BY4742 background was a kind gift from Weissman [6]. This strain was used in the present study to integrate a misfolded protein (DM-MBP, SM-MBP, oxDM-MBP 18, 296) expressed under an inducible *GAL1* promoter together with a Norceothrycin resistance marker at the *TEF2pr*-RFP-MET15-*URA3* locus as well at the *IRE1* gene locus. Gene disruption /integration was done by PCR based homology recombination method[5]. Disruption at *URA3* locus was confirmed by PCR using MBP specific oligonucleotides MBP_FOR and MBP_REV and streaking on minimal media plates lacking Uracil. Disruption at the *IRE1* locus was confirmed by *IRE1* luminal domain specific oligonucleotides IRELD_FOR and IRELD_REV.

Statistical Analysis : Mann Whitney test was performed using Wilcox program of R statistical analysis package (http://www.R-project.org/).

Galactose Induction assay

The yeast strains disrupted at the *URA3* locus alone, and *URA3*, *IRE1* locus together, were grown in YP 2% Raffinose media, overnight. These cultures were inoculated in the same media and allowed to grow till they reached the exponential phase. Cells were induced with 2% Galactose and grown for 10 hours (O.D_{600} not exceeding 2.0). To address the effect of TMAO on refolding the misfolded forms of MBP, 50mM, 100mM, 200mM, 500mM was added during galactose induction. 2mM DTT was added to cells and incubated for 2 hours in YP-2%Raffinose media alone in presence/absence of 200mM TMAO . Cells were washed with PBS and cells expressing the Green Fluorescent Protein (GFP) were quantitatively determined using FACS.

Immunoblot Analysis of S. Cerevisiae

Yeast cells corresponding to OD_{600} of 1.0 were pelleted and resuspended in SDSPAGE sample loading buffer containing SDS and β-ME. The cell pellet was migrated on 12% SDS-Polyacrylamide gel and transferred onto PVDF membrane using the standard western blot procedure. MBP expression was revealed with MBP specific antibody and chemiluminescence detection. The MBP expression levels were normalized to those of PGK (phosphoglycerate kinase).

TMAO estimation in *S. cerevisiae*

S. cerevisiae strain yMJ003 was grown at 30°C for 15 hrs in Synthetic Dextrose media with different concentrations of TMAO (50mM, 100mM, 200mM and 500mm). Cells were

harvested and then quickly washed twice with ice cold phosphate buffered saline. Cell pellets were weighed and resuspended in equal volume of water. 12% trichloroacetic acid (TCA) solution was added to the cell suspensions, vortexed and then centrifuged at 10,000g for 5 min at 4°C. TMAO estimation was performed as described previously[7] . Briefly, An aliquote of 100µl of the supernatant obtained for each sample and was used for TMAO estimation. 100µl of 0.1M ferrous sulphate in 0.1M EDTA in acetate buffer (pH7.4) was added to 100µl of TCA extracted supernatant. 300µl of toluene was added to it. The sample was then incubated at 50°C for 5 min. Samples were then allowed to cool at room temperature. 100µl of 45% KOH solution (w/v) was added and vortexed for 5 min. 200µl of toluene phase was taken and added to 650 µl of 0.2% picric acid (in toluene) and about 20mg of sodium sulphate. 200µl of the toluene phase was taken in a 96-well microtiter plate and spectrophotometric measurement was carried out at 410nm on a microplate reader (Tecan Infinite M200). Standard curve for TMAO was obtained by using known concentrations of TMAO which was then used to calculate intracellular TMAO concentrations.

Total intracellular carbohydrate estimation

E. coli cells were grown overnight at 37°C in LB without or with 200mM TMAO or 200mM Proline or 10% Glycerol. *S. cerevisiae* was grown similarly as mentioned in TMAO estimation protocol. Both *E. coli* and *S. cerevisiae* cells were pelleted, washed and extracted with TCA as described for TMAO estimaton protocol. 100µl of TCA extracted supernatant was diluted 5-fold with dist. water to 500 µl in a glass test tube and kept on ice. Carbohydrate estimation was performed as described previously[8] . Briefly, 1ml of chilled 75% H_2SO_4 solution was added and vortexed briefly. 2ml of chilled 0.2% anthron solution (prepared in 75% H_2SO_4) was added and vortexed. The samples were then boiled at 100°C for 15 min

followed by cooling to room temperature. 200µl of each sample was taken in a 96-well microtiter plate and absorbance was measured at 578 nm on microplate reader (Tecan Infinite M200). Standard curve was obtained by using known concentrations of trehalose which was then used to calculate intracellular carbohydrate concentrations for each growth condition. Intracellular glucose was estimated by peroxidise based assay using Roche diagnostic kit and subtracted from the estimated total-carbohydrate obtained from Antrone-assay to obtain the concentration of trehalose.

Table 1: Growth of different Glycine-duplet substitution mutants in presence of different osmolytes

Residues Mutated		Growth(normalized w.r.t WtGmR)				
Residue1	Residue2	LB	LB+TMAO	LB+Proline	LB+Glycerol	LB 30DC
5	6	0.258801731	0.8452	0.315605908	0.271817856	0
7	8	0	0	0	0	0
9	10	0	0	0	0	0
11	12	0	0	0	0	0
13	14	0	0	0	0	0
15	16	0	0	0	0	0
17	18	0	0	0	0	0
19	20	0	0	0	0	0
21	22	0.391599737	0.9375	0	0.257330116	0
23	24	0	0.744	0	0	0.811
25	26	0	1.0627	0	0	0
27	28	0	0.2764	0	0	0.8516
29	30	0	0	0	0	1.013
31	32	0	0	0	0	0
33	34	0	0	0	0	0
35	36	0	0	0	0	0
37	38	0	0.768	0	0	0
39	40	0	0	0	0	0.4299
41	42	0	0.214	0	0	0
43	44	0	0	0.286835237	0	0.3594
45	46	0	0	0	0	0.2902
47	48	0	0	0	0	0
49	50	0	0	0	0	0
51	52	0	0	0	0	0
53	54	0	0.2545	0	0	0
55	56	0	0.6984	0.34699215	0.375991725	0
57	58	0.230389107	0	0	0	0
59	60	0.479308208	0.9337	0.788142905	0.40703687	1.0442
61	62	0	0.2322	0	0	0.8381
63	64	0.269302034	0.8207	0.535309498	0.289755075	0
65	66	0	0.7188	0	0	0
67	68	0	0	0	0	0
69	70	0	0	0	0	0
71	72	0.29339097	0	0	0	0
73	74	0.21371219	0.7268	0	0	0
75	76	0.240271782	0	0	0	0.4231
77	78	0	0	0	0	0
79	80	0	0	0	0	0
81	82	0	0	0	0	0
83	84	0	0	0	0	0

85	86	0.209388503	0.8241	0.551874468	0.330458785	0
87	88	0	0	0	0	0
89	90	1.030265555	0.7473	1.04533564	0.565022375	0.7215
91	92	0.383570096	0	0	0	0.2631
93	94	0	0	0	0	0
95	96	0	0	0	0	0
97	98	0	0	0	0	0
99	100	0.229153802	0	0	0	0
101	102	0	0	0	0	0
103	104	0.288449655	0	0	0	0
105	106	0.375540448	0	0	0	0
107	108	0.396541053	0	0	0	0
109	110	0	0	0.414995611	0	0
111	112	0.2359481	0	0	0	0
113	114	0	0	0	0	0.811
115	116	0	0	0.367044469	0	0
117	118	0	0	0	0	0
119	120	0	0	0	0	0
121	122	0.420012361	0.7408	0.695727971	0.356674699	0
123	124	0	0.2619	0	0	0
125	126	0	0	0	0	0
127	128	0	0	0	0	0
129	130	0	0	0	0	0.5085
131	132	0	0	0	0	0
133	134	0	0	0	0	0
135	136	0	0.7289	0	0.249741265	0
137	138	0	0.6959	0	0.272507763	0
139	140	0	0	0	0	1.1188
141	142	0	0	0	0	0
143	144	0	0	0	0	0
145	146	0	0	0	0	0
147	148	0	0	0	0	0
149	150	0	0	0	0	0
151	152	0	0	0	0	0
153	154	0	0	0	0	0
155	156	0	0	0	0	0.4231
157	158	0	0	0	0	0
159	160	0.282272991	0	0	0	0
161	162	0.26744905	0.7511	0	0	0
163	164	0	0	0	0	0
165	166	0	0	0	0	0
167	168	0	0	0	0	0
169	170	0	0	0	0	0
171	172	0	0	0	0	0

173	174	0	0	0	0	0
175	176	0	0	0	0	0

Table 2: Activity of CcdB mutants

Clone no.	Activity			
1	LB	LB+TMAO	LB+Proline	LB+Glycerol
2	0	0	0.000370459	0.087904663
3	0	0.036118094	0.012812596	0.076027159
4	0	0.158181179	0.130278587	0.122388916
5	0.014566671	0.039572881	0	0.089615033
6	0.015686272	0.099494814	0.116352231	0.158000097
7	0.04248607	0.099560259	0.031953375	0.063086337
8	0.043106018	0.016660854	0	0.039863727
9	0.043137222	0.03575595	0.151841893	0.158901664
10	0.043137222	0.2697241	0.111859791	0.115627317
11	0.045172735	0.03193338	0.327899203	0.115843299
12	0.053092007	0.109988405	0.157681929	0.182341929
13	0.059125201	0.238243327	0	0.14177221
14	0.060331768	0.095608234	0.113656763	0.091285485
15	0.066666674	0.139525828	0.168912895	0.127798232
16	0.069677162	0.09830405	0.04250032	0.089291458
17	0.070891034	0.065012589	0.017890784	0.141701701
18	0.072096541	0.107656458	0.219227341	0.210740735
19	0.073906435	0.049747407	0.109164459	0.146279964
20	0.075503773	0.08825372	0	0.11549658
21	0.078129735	0.336572109	0.219227341	0.209388389
22	0.079336303	0.109599687	0.17250678	0.110218
23	0.082352945	0.090944453	0.128930788	0.122839695
24	0.085972825	0.067625323	0.056603795	0.020063119
25	0.085972825	0.424795959	0.162623546	0.059280558
26	0.089827627	0.086997511	0.074922303	0.082174095
27	0.09104157	0.125628117	0.106563196	0.143319281
28	0.091101072	0.171006601	0.117250717	0.078663853
29	0.09419762	0.137248777	0.107344466	0.120349414
30	0.095168746	0.065954768	0.083516138	0.075056632
31	0.095654311	0.064698468	0.142891524	0.036234264
32	0.096625435	0	0	0.070203801
33	0.098567615	0.109296462	0.168672962	0.098997123
34	0.098642544	0.214146938	0.113656763	0
35	0.100024303	0.1350502	0.180391749	0.045616354
36	0.100267122	0.118718547	0.190157428	0.10449691
37	0.104977356	0.198989511	0.217430364	0.258973616
38	0.105278976	0.052467955	0.078167101	0
39	0.106822041	0.138190957	0.171407342	0.085085774
40	0.107064859	0.202889422	0.002265651	0.092203141
41	0.107088961	0.007773043	0.111410613	0.25762127
42	0.108295618	1	0.07187782	0

43	0.109735417	0.137562806	0.13820403	0.076027159
44	0.11094929	0.113065278	0.106172564	0.07311548
45	0.111915501	0.109599687	0.120395357	0.245901137
46	0.113374651	0.105866	0.367543524	0.128790505
47	0.117504281	0.126256267	0.217891979	0.070527373
48	0.117803379	0.187087825	0.135235246	0.081771784
49	0.120165304	0.08989936	0.116709884	0.081090339
50	0.121266955	0.097940181	0.19991019	0.264382932
51	0.122602584	0.392902021	0	0
52	0.12488924	0.128080571	0.238236393	0.014991533
53	0.127700957	0.065012589	0.162032299	0.112908475
54	0.130128702	0.105841678	0.088203745	0.152377893
55	0.131342571	0.144472376	0.135078959	0.09285019
56	0.131523359	0.184998058	0.066487025	0.09534246
57	0.134238294	0	0.191823932	0.310361904
58	0.136048279	0.082394095	0.268194056	0.255818208
59	0.136651606	0.111542921	0.122641507	0.214346922
60	0.137858174	0.195491649	0.181042279	0.221108522
61	0.138763211	0.144578323	0.157681929	0
62	0.139839816	0.119032668	0.124532073	0.15723063
63	0.152464234	0.158605548	0.108125674	0.133937285
64	0.15270698	0.161746211	0.16554795	0.096732494
65	0.153544445	0.158181179	0.181042279	0.203979136
66	0.153544445	0.048192774	0.060197748	0
67	0.15659141	0.102701016	0.129610199	0.169200924
68	0.161689786	0.161432182	0.074141154	0.128760879
69	0.164117528	0.146670859	0.083125561	0.130054981
70	0.166817492	0.10104933	0.255166249	0.357693222
71	0.168001958	0.219535154	0.244845323	0.064704014
72	0.169230714	0.125534374	0.346810465	0.364004037
73	0.170135752	0.301204815	0.145103371	0.121487417
74	0.17334308	0.155464792	0.264376675	0.079262413
75	0.173828642	0.230527613	0.297970619	0.132643186
76	0.174785913	0.163832032	0.170803968	0.034412289
77	0.179509852	0.160361008	0.1674694	0
78	0.179788829	0.467547638	0.34636122	0.236885629
79	0.184615366	0.327244492	0.16172506	0
80	0.186123643	0.176447696	0.125786148	0.117881162
81	0.188152497	0.14038944	0.191329329	0.123584571
82	0.189609185	0.161118061	0.132344579	0.109026169
83	0.192795949	0.298160385	0.303445727	0.109028976
84	0.193008054	0.130339207	0.206563827	0.152054321
85	0.1954751	0.129420954	0.247079991	0.28917548
86	0.196078428	0.1923825	0.303234541	0.260325962
87	0.197815181	0.283582075	0.257873296	0.126405507

88	0.198700891	0.137452272	0.290477965	0.073594574
89	0.199320226	0.277010063	0.245626531	0.158201254
90	0.202233606	0.310615554	0.382736753	0.141054651
91	0.206636452	0.266226238	0.211141083	0.262129024
92	0.207239779	0.222697241	0.293800556	0.145829181
93	0.209034564	0.163832032	0.094479435	0.157069861
94	0.211459085	0.179020046	0.141329045	0.130378556
95	0.213401335	0.143530102	0.174532357	0.148495589
96	0.2189852	0.26853016	0.323361426	0.14623096
97	0.222626887	0.235552776	0.242501515	0.133290235
98	0.228657626	0.248736918	0.376010784	0.264382932
99	0.236500715	0.193937074	0.181940698	0.27249681
100	0.23921565	0.095608234	0.208894866	0
101	0.241327255	0.181111595	0.18733156	0
102	0.242232293	0.40730664	0.349056615	0
103	0.246455502	0.15856978	0.311769976	0.14943544
104	0.247360449	0.092499085	0.28706203	0
105	0.248868724	0.337738083	0.153638802	0.280610755
106	0.252974029	0.160804032	0.19406371	0.13458424
107	0.257918561	0.330353699	0.325247092	0.309460341
108	0.258220181	0.160901727	0.178346816	0.17512955
109	0.259043456	0.225502497	0.372580443	0.089615033
110	0.259728459	0.219199379	0.334231836	0.317123503
111	0.263046718	0.067625323	0.238993738	0.211191514
112	0.270454006	0.307160814	0.208126301	0.11549658
113	0.270939568	0.27104272	0.29640814	0.188288585
114	0.271794848	0.144189726	0.295597528	0
115	0.271794848	0.310921091	0.211141083	0.176031117
116	0.272639003	0.152010005	0.220235783	0.153348423
117	0.273906453	0.23591138	0.345462734	0.321631257
118	0.276645976	0.182922587	0.218599486	0.312776839
119	0.279336317	0.176447696	0.350853592	0.477599251
120	0.281369956	0.189517526	0.270470547	0.215672953
121	0.282846171	0.188823312	0.28566134	0.110732533
122	0.284162857	0.169840685	0.307277672	0.051617329
123	0.288687774	0.579090562	0.384546282	0.696675639
124	0.288989394	0.48892342	0.462264138	0.312164966
125	0.296132271	0.195418251	0.357539815	0.109028976
126	0.301741961	0.311350214	0.333086288	0.184327107
127	0.314932158	0.273610563	0.292902066	0.148083094
128	0.315913771	0.249913211	0.303075216	0.103918209
129	0.316338945	0.252512538	0.319845779	0.174377233
130	0.320708938	0.278894473	0.364767937	0.278550619
131	0.323981904	0.113486267	0.319407056	0.063788245
132	0.325791802	0.184998058	0.186433069	0.137264524

133	0.328477801	0.281721106	0.33664275	0.114202574
134	0.329495121	0.233599466	0.457947365	0.319591162
135	0.330920049	0.178002329	0.372866143	0.203528353
136	0.336953244	0.254566673	0.311320799	0.09534246
137	0.341009756	0.387018426	0.419414585	0.267802393
138	0.342485968	0.451926401	0.015561253	0.181942109
139	0.3502262	0.40536341	0.529200341	0.194062066
140	0.352270024	0.30841707	0.47062796	0.215464233
141	0.355656065	0.251068807	0.340521117	0.256719707
142	0.355911636	0.421482392	0.314767595	0.166289245
143	0.368627404	0.228138395	0.426774471	0
144	0.377029827	0.320721964	0.277880658	0.111073303
145	0.377325077	0.290524123	0.330492745	0.191141431
146	0.384410982	0.397431445	0.319007036	0.251448077
147	0.393563604	0.269698032	0.373471639	0.294037473
148	0.400059048	0.365151008	0.33530937	0.244974475
149	0.404826527	0.460940572	0.495507651	0.275652286
150	0.408621212	0.251648747	0.310114868	0.130153391
151	0.408916421	0.287400208	0.246758046	0.410221494
152	0.40921167	0.287053099	0.314931437	0.314480447
153	0.451282057	0.424018645	0.905211152	0.958575622
154	0.460030138	0.310921091	1	0.99103139
155	0.469441972	0.348837211	0.340496454	0.418398652
156	0.498376138	0.523429371	0.315672467	0.276320274
157	0.506052545	0.473099601	0.236013306	0.343441243
158	0.538234435	0.493925748	0.041126322	0.071891017
159	0.54871794	0.465993009	0.646451053	0.746711617
160	0.55294115	0.590361468	0.658580437	0.828752585
161	0.557767732	0.408861272	0.547169828	0.455060481
162	0.571945681	0.350174907	0.533243468	0.199922166
163	0.58883861	0.543334667	0.678796079	0.8476851
164	0.600603289	0.691799457	0.693620859	0.54521543
165	0.61647477	0.49461991	0.416821043	0.679386725
166	0.635595757	0.612125906	0.747529223	0.859405232
167	0.654298623	0.670812275	0.85579516	0.816581669
168	0.814585182	0.848663661	0.828825487	0.847700173
169	0.830823733	0.851440467	0.835124113	0.855536633
170	0.838500152	0.812217986	0.795109291	0.82453152
171	0.841747863	0.838250613	0.840311227	0.850766613
172	0.845881306	0.836515101	0.824749903	0.838160143
173	0.847062292	0.834432494	0.82734346	0.841567291
174	0.84942427	0.833044091	0.806224527	0.81873936
175	0.84942427	0.848663661	0.824749903	0.82555367
176	0.850605254	0.839639016	0.828825487	0.841908013
177	0.85178624	0.837903505	0.824379392	0.840204433

178	0.853852966	0.803887545	0.817710266	0.833390119
179	0.855919694	0.836515101	0.82104482	0.841567291
180	0.855919694	0.851440467	0.824749903	0.851107335
181	0.857100677	0.838250613	0.826602445	0.842589441
182	0.857100677	0.83755641	0.824749903	0.839863724
183	0.85769117	0.833391186	0.802148943	0.838160143
184	0.859757899	0.850052064	0.825861434	0.846337311
185	0.859757899	0.836167993	0.826602445	0.840545151
186	0.859757899	0.837903505	0.826972945	0.839863724
187	0.86005314	0.838250613	0.82215635	0.840545151
188	0.86005314	0.853870183	0.836606143	0.852810902
189	0.86064363	0.832002779	0.815857725	0.840204433
190	0.860938882	0.84588685	0.807706557	0.838841571
191	0.861234135	0.828531755	0.809188584	0.833049411
192	0.861529375	0.837903505	0.828084476	0.839863724
193	0.863005611	0.851093372	0.838458685	0.848381601
194	0.863005611	0.841374528	0.835124113	0.849744463
195	0.863073564	0.858040196	0.87961488	0.841151732
196	0.863300852	0.84588685	0.824749903	0.848040892
197	0.864777087	0.849704969	0.833642086	0.848381601
198	0.865072337	0.850746267	0.819933308	0.851448044
199	0.865072337	0.829920172	0.816228225	0.839523002
200	0.865367577	0.852134684	0.824379392	0.851788763
201	0.86595807	0.832349874	0.82104482	0.837819421
202	0.867139054	0.820201318	0.818451278	0.832708692
203	0.867434306	0.852828884	0.826231934	0.84667802
204	0.867434306	0.849704969	0.826231934	0.828620113
205	0.867434306	0.847275254	0.829195987	0.850085182
206	0.867729559	0.826796257	0.824008892	0.838160143
207	0.868320049	0.825754949	0.816228225	0.841226582
208	0.868910542	0.832002779	0.825120404	0.841567291
209	0.869205782	0.84380423	0.819933308	0.835775131
210	0.869796275	0.832696983	0.82215635	0.836797281
211	0.870091525	0.852481779	0.817339751	0.845996593
212	0.870091525	0.846928149	0.835494628	0.852470194
213	0.870091525	0.813953498	0.828454976	0.841226582
214	0.870386766	0.851787576	0.836235643	0.853492333
215	0.871085217	0.866206035	0.888989934	0.837593013
216	0.871272511	0.848316566	0.828825487	0.841908013
217	0.871272511	0.852828884	0.835494628	0.851788763
218	0.871567761	0.826102054	0.824749903	0.839523002
219	0.871863001	0.82818466	0.813264168	0.839182279
220	0.872158254	0.850052064	0.841052238	0.849403754
221	0.872453494	0.847275254	0.828084476	0.841226582
222	0.872453494	0.796945511	0.831048529	0.848040892

223	0.872748747	0.836167993	0.788440151	0.838160143
224	0.872748747	0.84033322	0.812152641	0.849063032
225	0.873339237	0.853870183	0.835494628	0.842248722
226	0.87392973	0.847275254	0.839199696	0.849744463
227	0.874224982	0.850052064	0.831048529	0.848722323
228	0.874241323	0.84359296	0.885083652	0.846651567
229	0.874241323	0.86463568	0.888599303	0.852798451
230	0.874520223	0.84588685	0.835124113	0.852129472
231	0.874726875	0.858668334	0.8800055	0.841798768
232	0.875697992	0.864321603	0.881177386	0.85700421
233	0.875996459	0.851440467	0.838458685	0.851448044
234	0.875996459	0.850052064	0.833642086	0.850766613
235	0.876911874	0.867462313	0.877271119	0.850210286
236	0.87909687	0.857726132	0.823755157	0.843092848
237	0.879339652	0.862751261	0.889771189	0.852151412
238	0.881767421	0.865263817	0.880396131	0.844063411
239	0.881767421	0.866206035	0.89172433	0.854416041
240	0.882010203	0.866206035	0.889771189	0.85700421
241	0.882495755	0.861180906	0.875708598	0.847945656
242	0.882495755	0.867776377	0.882739892	0.84762213
243	0.882738528	0.853015077	0.883130527	0.848592693
244	0.882738528	0.865577881	0.881958641	0.851827888
245	0.884680754	0.864007539	0.866333539	0.850210286
246	0.884923527	0.864007539	0.889771189	0.852798451
247	0.884923527	0.866834172	0.871021076	0.851180849
248	0.885409079	0.866206035	0.874536723	0.854739567
249	0.886622968	0.866206035	0.890161825	0.833387254
250	0.887108523	0.854585422	0.887818048	0.855063094
251	0.887108523	0.863065325	0.886646174	0.853121965
252	0.887836857	0.866206035	0.889380554	0.85700421
253	0.887836857	0.865891958	0.886255542	0.852474925
254	0.88807963	0.86149497	0.890552444	0.855063094
255	0.88807963	0.866520099	0.885083652	0.852474925
256	0.888322412	0.863693462	0.888989934	0.854416041
257	0.888322412	0.855527636	0.892505585	0.853769005
258	0.888565194	0.862123108	0.894458726	0.854739567
259	0.888807967	0.865891958	0.89211495	0.852151412
260	0.888807967	0.863693462	0.883521146	0.852798451
261	0.889050747	0.861180906	0.890161825	0.852151412
262	0.889050747	0.862437184	0.888599303	0.841798768
263	0.889050747	0.860866829	0.885083652	0.857651246
264	0.889050747	0.866834172	0.866333539	0.851180849
265	0.889293519	0.867148236	0.887427429	0.854092528
266	0.889536301	0.858040196	0.888599303	0.848592693
267	0.889779074	0.869032655	0.89211495	0.848592693

268	0.890021856	0.839195981	0.874536723	0.849886772
269	0.890264629	0.865263817	0.887427429	0.855386607
270	0.890264629	0.864949744	0.887427429	0.854739567
271	0.890750181	0.865891958	0.890552444	0.804593977
272	0.892206852	0.865891958	0.884302401	0.853769005
273	0.952941173	0.998056739	1	0.986974429
274	0.963197586	0.991061026	0.992362979	0.976155841
275	0.963499239	0.75553829	1	0.983819003
276	0.98069381	0.994558889	1	0.99103139
277	0.982805427	0.996502138	1	0.99103139
278	0.983107089	0.969685201	1	0.986974429
279	0.984313725	0.994947532	1	0.99103139
280	0.985218694	0.999222697	1	0.99103139
281	0.986425339	0.998445398	1	0.98607288
282	0.986425339	1	1	0.925218318
283	0.987028657	0.998834054	1	0.99103139
284	0.988235291	0.996890793	1	0.98111436
285	0.988536944	0.995724835	1	0.99103139
286	0.996078425	0.997668096	1	0.99103139
287	0.999698341	1	1	0.984269768
288	1	0.997668096	1	0.99103139

Table 3: List of Yeast Strains

Strain	Ref
YMJ003 MATα *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 LYS+Δcan1::STE2*pr-*spHIS5Δlyp1:: STE3*pr-*LEU2 cyh2Δura3*::UPRE-GFP-*TEF2*pr-RFP-*MET15-URA3*	[6]
YNK001 (*URA3* disrupted with SM-MBP) MATα *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 LYS+Δcan1::STE2*pr-*spHIS5Δlyp1:: STE3*pr-*LEU2 cyh2Δura3*:: UPRE-GFP-*TEF2*pr-RFP-*MET15*-NAT-*GAL1pr-er*SM-MBP	This study
YNK002 (*URA3* disrupted with DM-MBP) MATα *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 LYS+Δcan1::STE2*pr-*spHIS5Δlyp1::STE3*pr-*LEU2 cyh2Δura3*:: UPRE-GFP-*TEF2*pr-RFP-*MET15*-NAT-*GAL1pr-er*DM-MBP	This study
YNK003 (*URA3* disrupted with oxDM-MBP 18,296) MATα *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 LYS+Δcan1::STE2*pr-*spHIS5Δlyp1::STE3*pr-*LEU2 cyh2Δura3*:: UPRE-GFP-*TEF2*pr-RFP-*MET15*-NAT-*GAL1pr-er*DM-MBP(18,296)	This study
YNK004 YNK002, *Δire1:KanMx*	This study

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Figure 1

MBP crystal structure (1omp) showing DM-MBP mutations

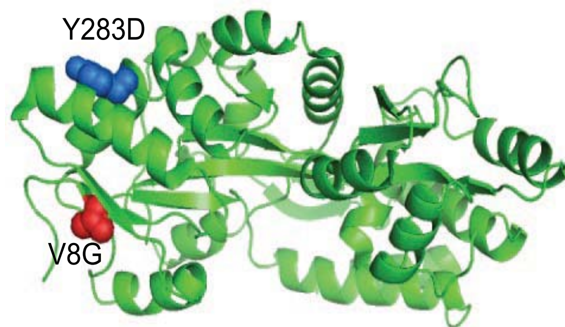


Figure 2

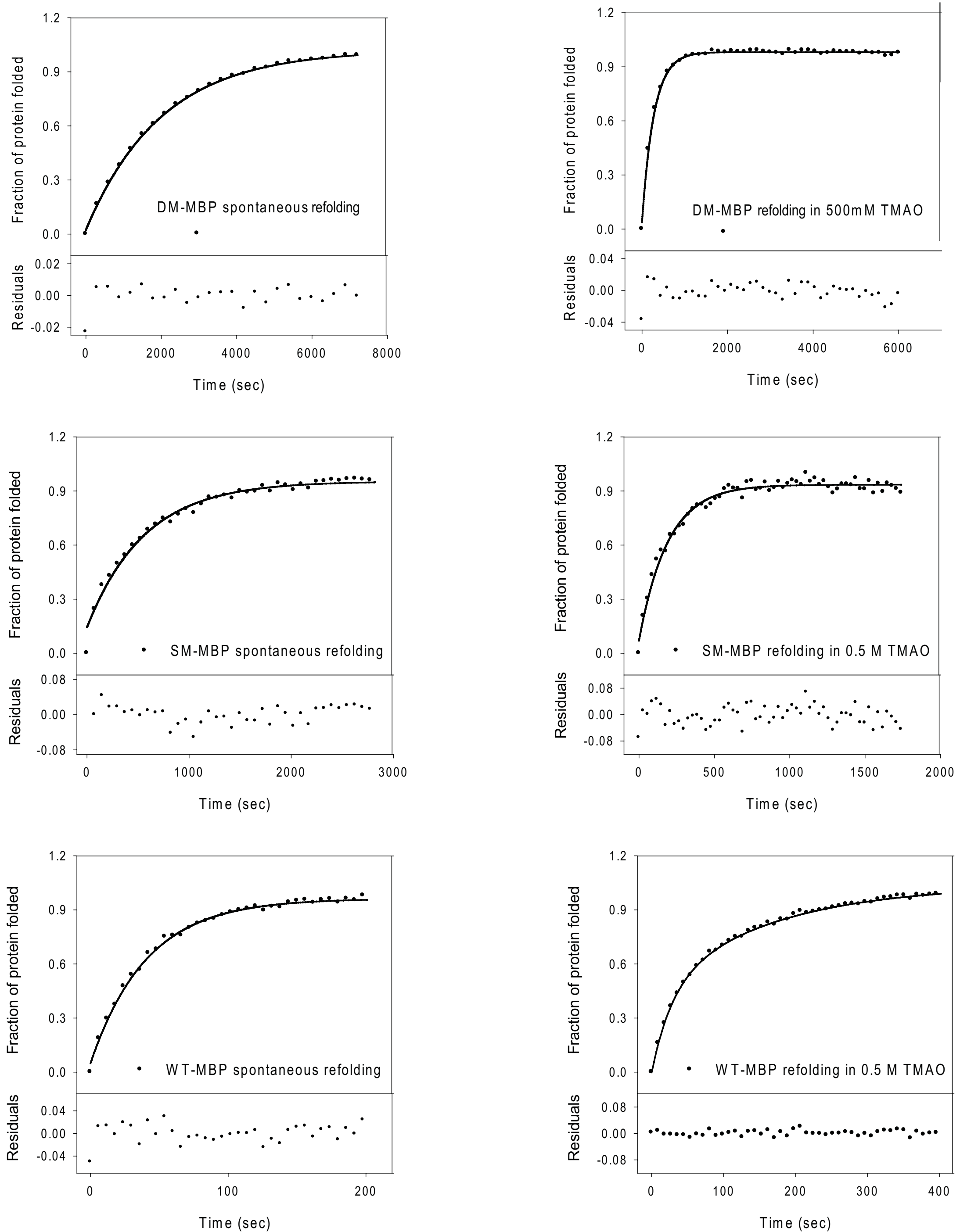
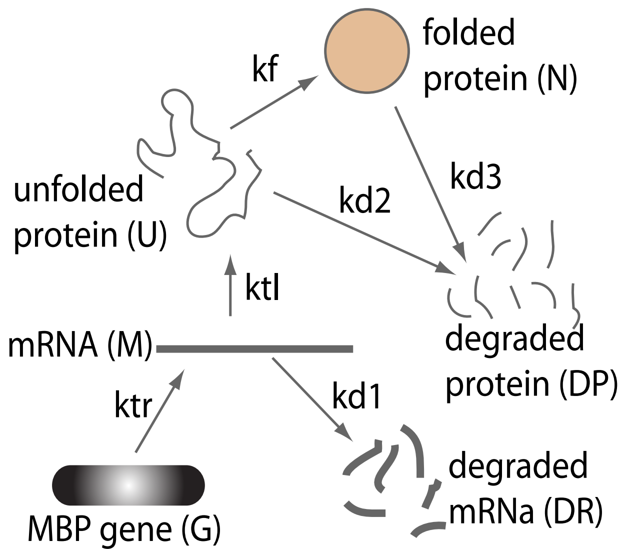


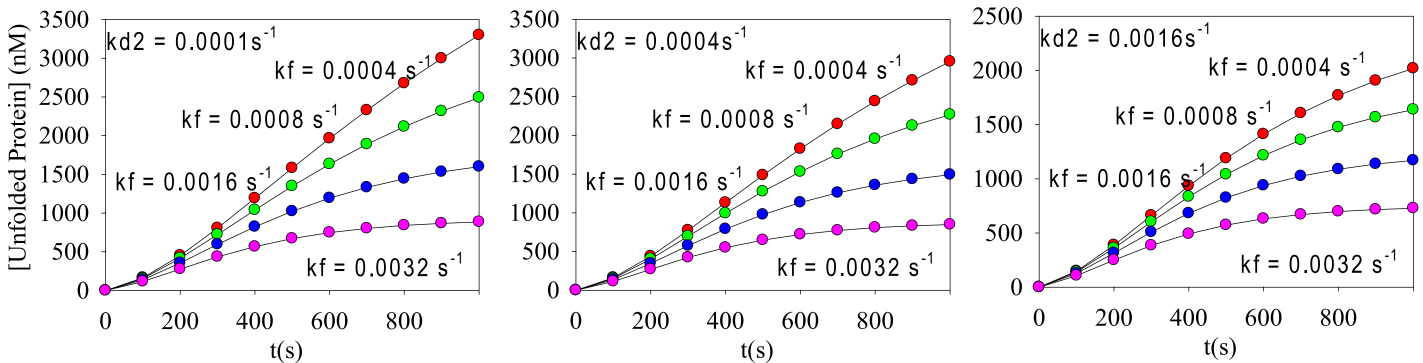
Figure 2 Legend

Non-linear regression based exponential fitting of folding rates of DM-MBP, SM-MBP and Wt-MBP in presence and absence of TMAO as indicated in the panels. The residuals are plotted to indicate the goodness of fit. The folding of wt-MBP deviates from single exponential behaviour in presence and absence of TMAO. This does not affect the conclusion as there is no obvious acceleration in folding rate afforded by TMAO. Both the spontaneous and the TMAO assisted refolding traces of Wt-MBP can be fit better using a bi-exponential function. Incorporation of 2 rate constants in both the cases does not alter the results. There is no rate acceleration with TMAO. Similarly the unfolding rate of SM-MBP deviates from single-exponential behaviour. But this does not affect the interpretation or conclusion of the section.

Figure 3

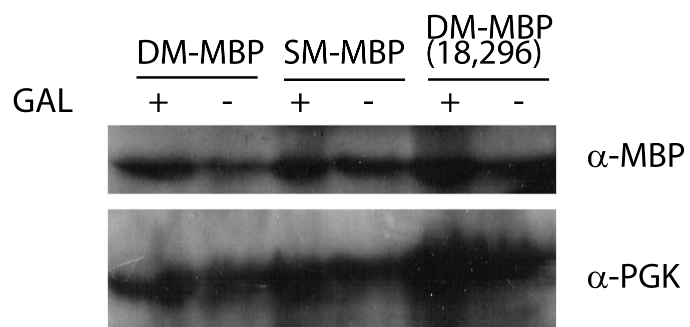


Parameter	Value	Unit
[G]	1	cell $^{-1}$
ktr	0.03	s^{-1}
ktl	0.01	s^{-1}
kf	Variable	s^{-1}
kd1	.25	s^{-1}
kd2	Variable	s^{-1}
kd3	0.25*kd2	s^{-1}



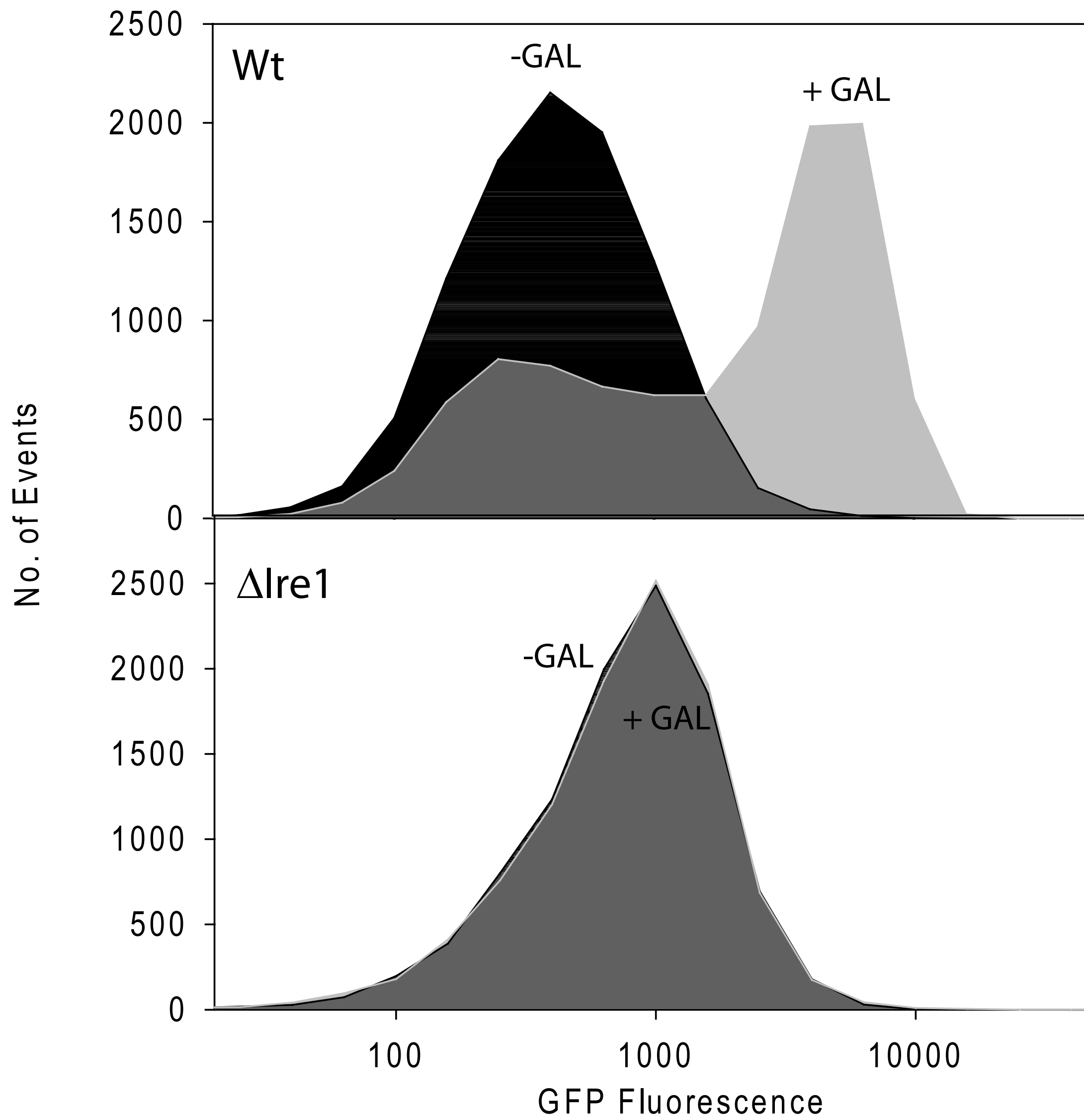
Numerical simulation to observe the effect of folding rate on steady-state concentration of non-native protein. Here the parameters were fixed as described. The rates of transcription and translation were kept fixed as the variants of DM-MBP described here does not exhibit altered expression as seen with western blot. It is also observed that under the various conditions tested, amount of DM-MBP produced did not alter significantly further justifying this assumption. We fixed these values to approximately the time required for transcription (assumed rate is 30 nucleotide/s) and translation (assumed rate is 4 aa/s). The degradation rate of mRNA was fixed at half the value of translation rate. Folding rate was varied from 0.0004s^{-1} to 0.1024 s^{-1}. Degradation rate was varied from 0.0001 to 0.002 s-1. The accumulation of non-native protein with time is shown for different folding rates and for different rates of degradation of the non-native protein.

Figure 4



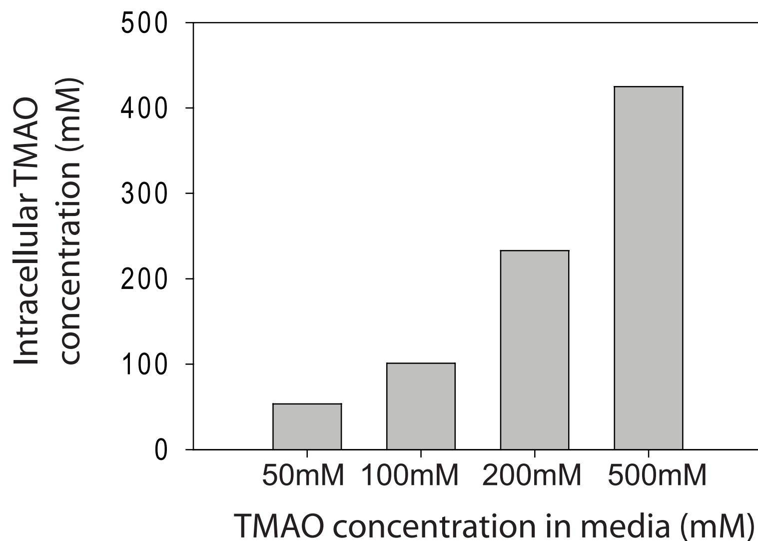
Expression level of DM-MBP, SM-MBP and DM-MBP(18,296) was checked using Western blotting with anti-MBP antibody. The blot was reprobed for PGK to control for the amount of loading.

Figure 5



A strain with genome integrated version of DM-MBP targeted to the ER and under the control of GAL-promoter was generated on the background of a strain containing UPRE-GFP and a deletion of IRE1. Cells were grown for induction as described previously and the fluorescence was recorded using a flow cytometer.

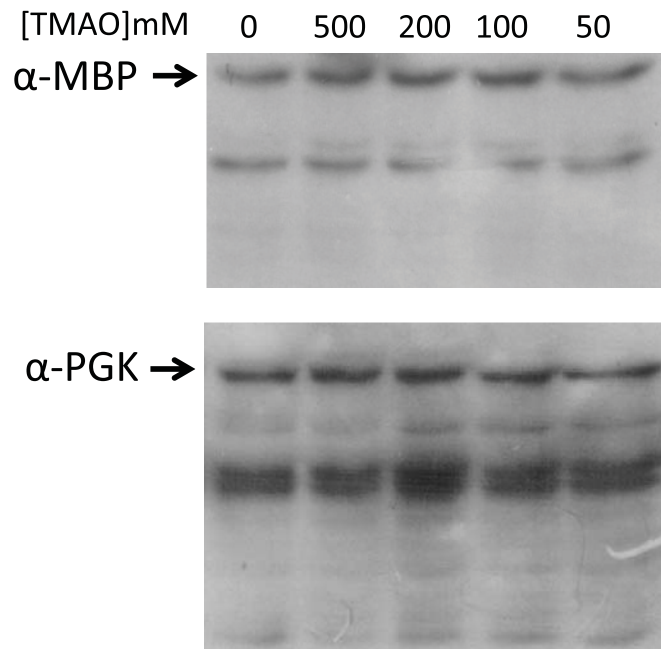
Figure 6



S. cerevisiae strain yMJ003 was grown at 30 °C for 15 hrs in Synthetic Dextrose media with different concentrations of TMAO (50mM, 100mM, 200mM and 500mm). Cells were harvested and then quickly washed twice with ice cold phosphate buffered saline. Cell pellets were weighed and resuspended in equal volume of water. 12% trichloroacetic acid (TCA) solution was added to the cell suspensions, vortexed and then centrifuged at 10,000g for 5 min at 4 °C. An aliquote of 100μl of the supernatant obtained for each sample and was used for TMAO estimation. 100μl of 0.1M ferrous sulphate in 0.1M EDTA in acetate buffer (pH7.4) was added to 100μl of TCA extracted supernatant. 300μl of toluene was added to it. The sample was then incubated at 50° C for 5 min. Samples were then allowed to cool at room temperature. 100μl of 45% KOH solution (w/v) was added and vortexed for 5 min. 200μl of toluene phase was taken and added to 650 μl of 0.2% picric acid (in toluene) and about 20mg of sodium sulphate. 200μl of the toluene phase was taken in a 96-well microtiter plate and spectrophotometric measurement was carried out at 410nm on a microplate reader (Tecan Infinite M200). Standard curve for TMAO was obtained by using known concentrations of TMAO which was then used to calculate intracellular TMAO concentrations.

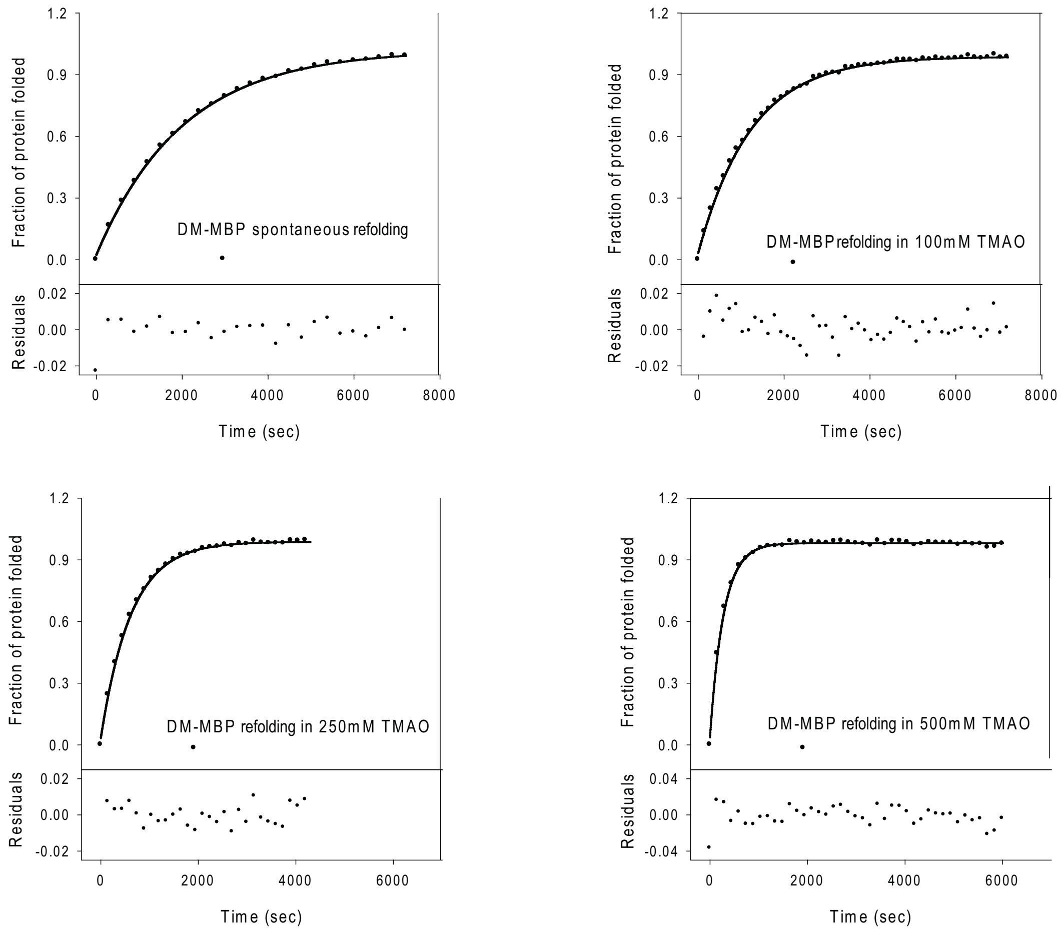
Figure 7

DM-MBP expression levels in S. cerevisiae



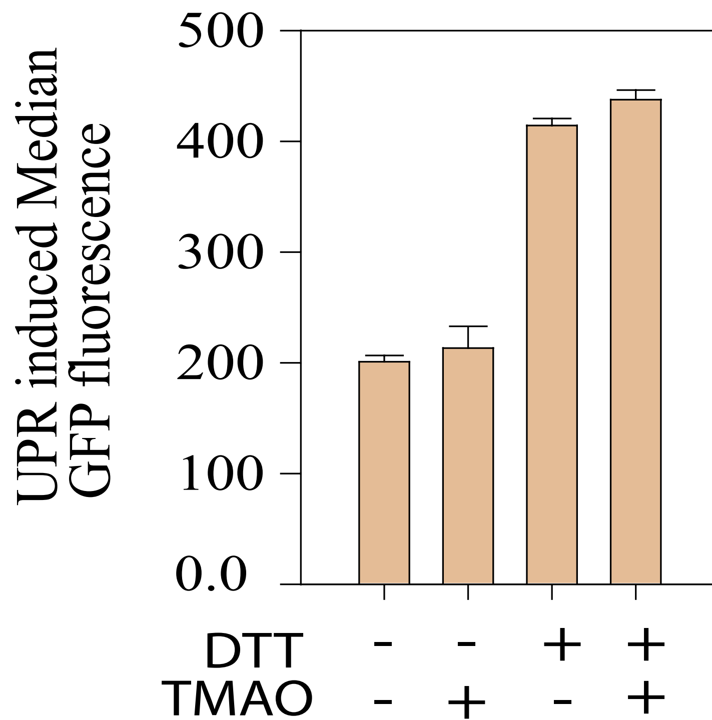
Protein expression level of DM-MBPO induced in 2% galactose containing YP media in presence of different concentrations of TMAO was determined by immunoblotting using MBP-specific antibody(upper blot). Lower bands correspond to the degradation product due to ER associated degradation. The expression level og yeast phospho-glycerate kinase was taken as the loading control (lower blot). PGK-band is shown with an arrow, the other bands are cross-reacting species.

Figure 8



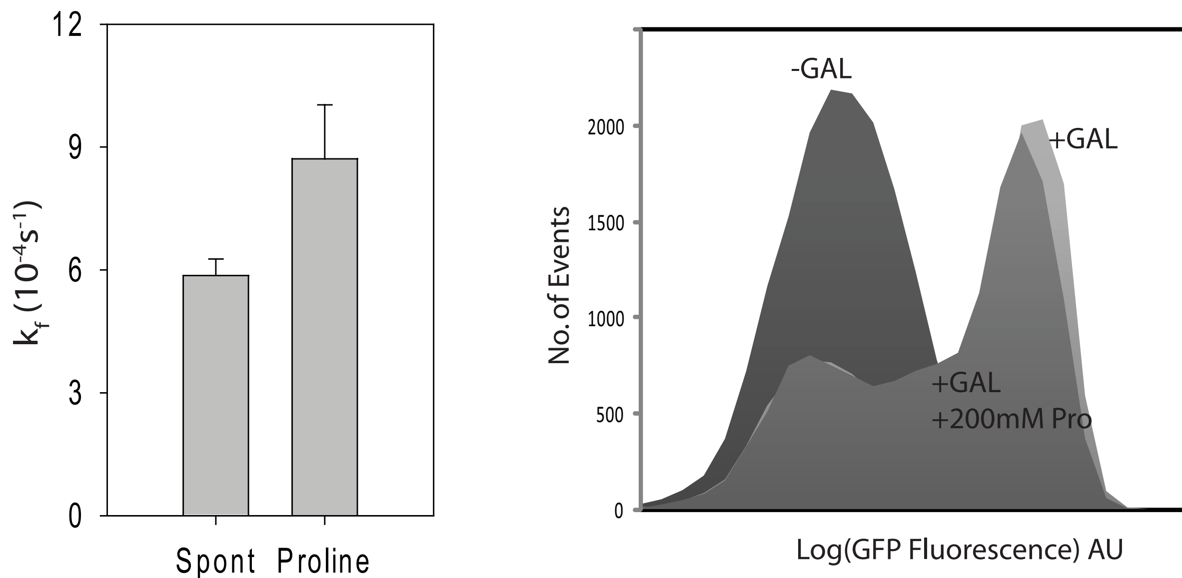
Non-linear regression based exponential fitting of DM-MBP folding rates in presence of different concentrations of TMAO as indicated in the panels. solid lines show the fitted curve. The residuals are plotted to indicate the goodness of fit. Some of the plots could be better fit to double exponential equation yielding better distribution of residuals but the other rate contributed insignificant amplitude to the fitting results. Thus single exponential fitting was deemed more appropriate for analysis.

Fig 9



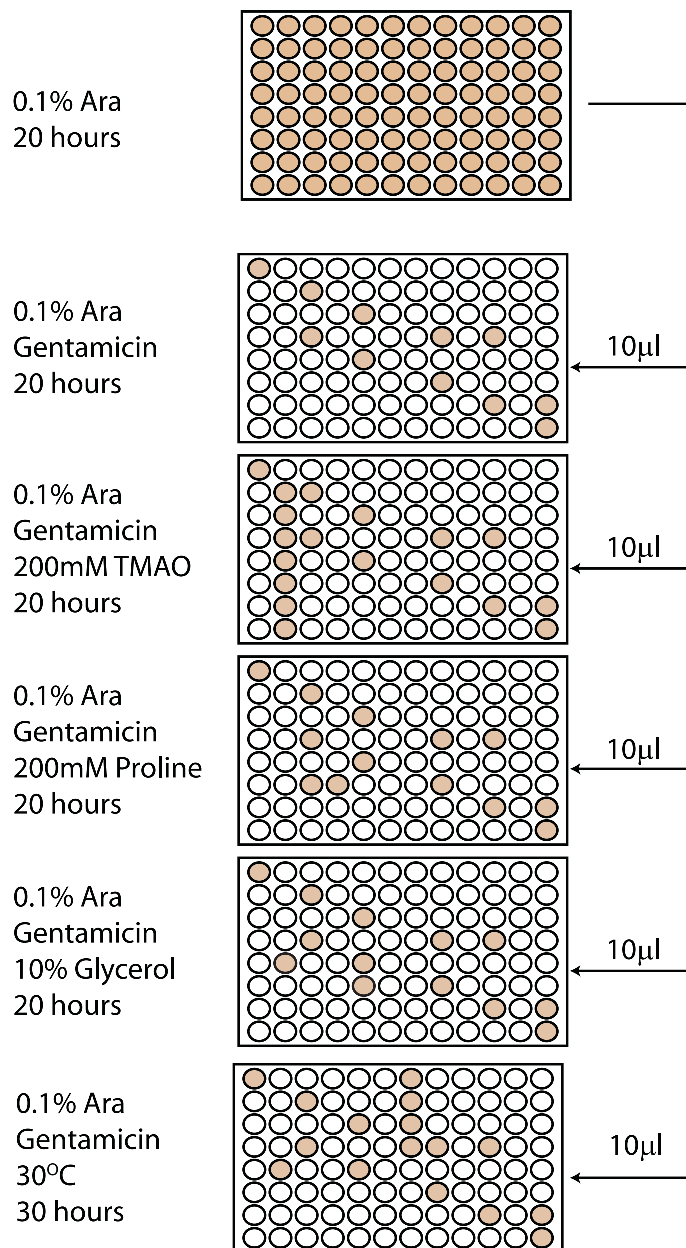
UPR was induced by addition of 5mM of DTT to S. ceresiae cells grown till the O.D 600 of 0.5. Fluorescence of GFP expressed under the control of UPRE is read out in a fluorimeter. No decrease in fluorescence upon addition of TMAO to a DTT treated sample rules out the possibility that TMAO affects the general UPR pathway. TMAO alone does not induce any UPR, thus chaperone content of the ER is not altered in presence of TMAO.

Figure 10



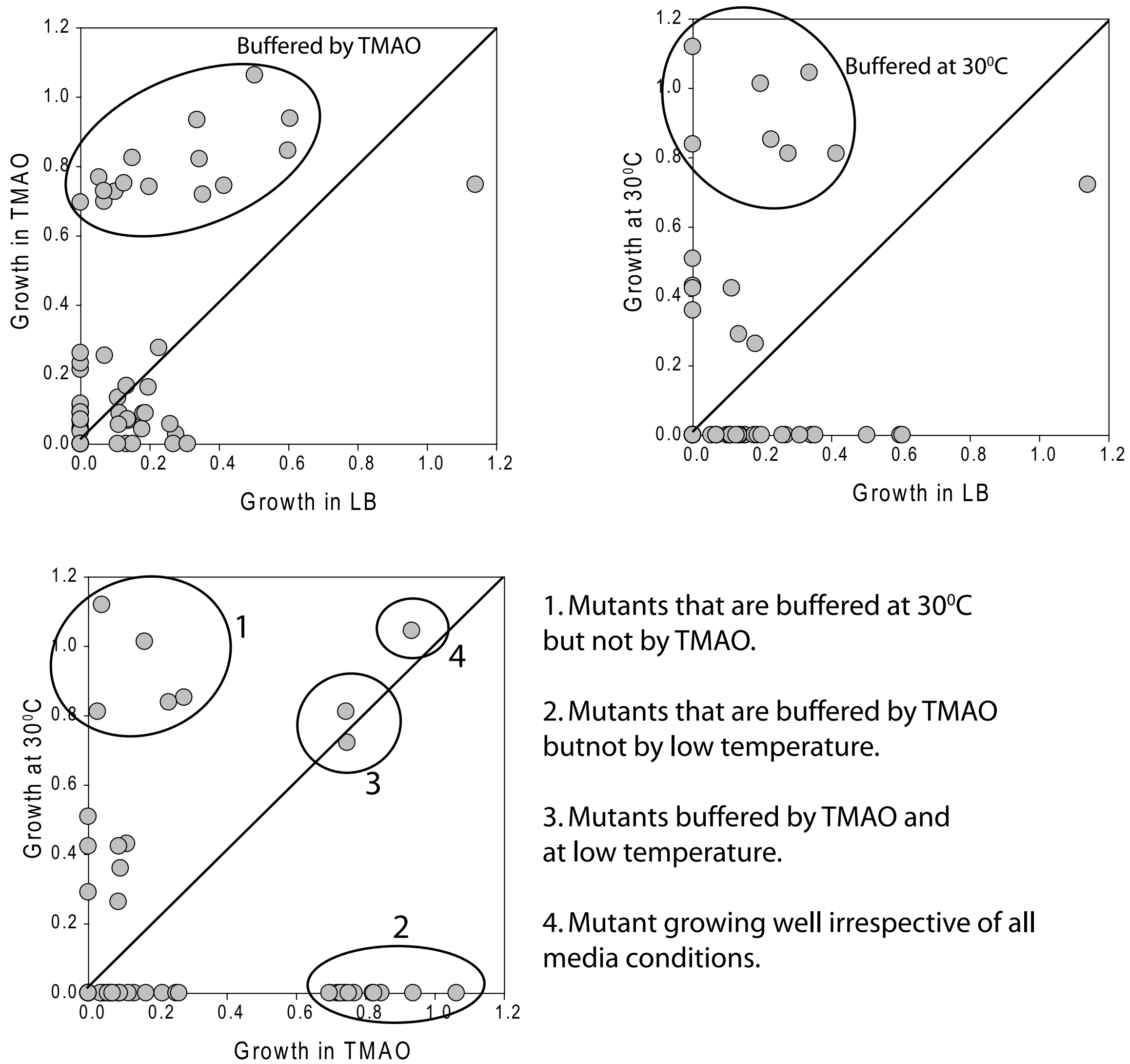
Left Panel: Refolding of DM-MBP was performed as described earlier in the presence of 200mM of Proline in the refolding buffer. Acceleration afforded by Proline is only marginal. Right Panel: Flowcytometric analysis of DM-MBP folding assistance in vivo in presence of Proline. S. cerevisiae was grown in 200mM of Proline during induction with Galactose and the decrease in UPR was recorded as the decrease in GFP fluorescence. There is a marginal, but reproducible, decrease of DM-MBP induced UPR in presence of Proline.

Figure 11



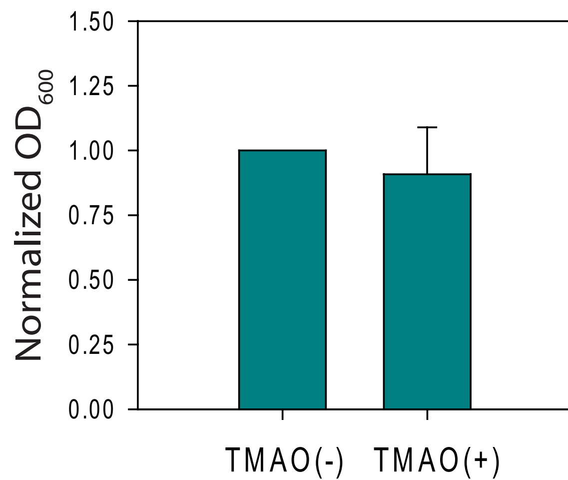
Characterization of Gentamycin glycine duplet substitution library: Schematic of the screening procedure for obtaining mutations buffered under different conditions. C) ΔΔG values due to mutations were calculated using FoldX. The ΔΔG values were calculate for all the mutants made, and the ΔΔG values for the different subsets are shown. One subset consists of the mutants that are more active in presence of 500mM TMAO than in its absence and the other subset consist of mutants that are more active at 30°C than at 37°C. D) In yellow are shown the residues which when mutated to glycine-duplets lead to proteins that are more active at 30°C than at 37°C.

Figure 12



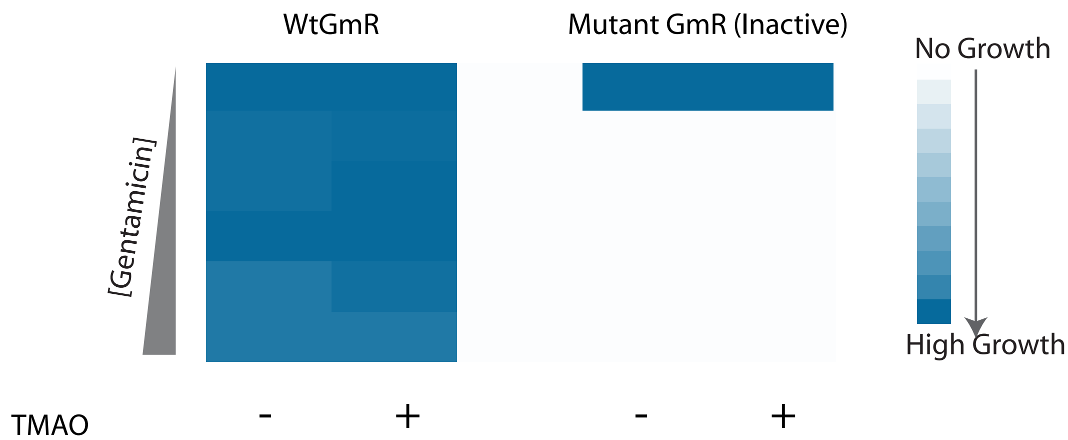
Growth of the different mutants are plotted against growth in presence of osmolyte or TMAO to obtain the mutants that are buffered at the respective conditions. Growth was measured as OD at 600nm and normalized with respect to the growth of WtGmR at each condition. Diagonal shows the expected trend for mutants that are equally active at both the growth conditions ploted in the X and Y axis. Mutations that are significantly off the diagonal are buffered and are circled for demarcation.

Figure 13



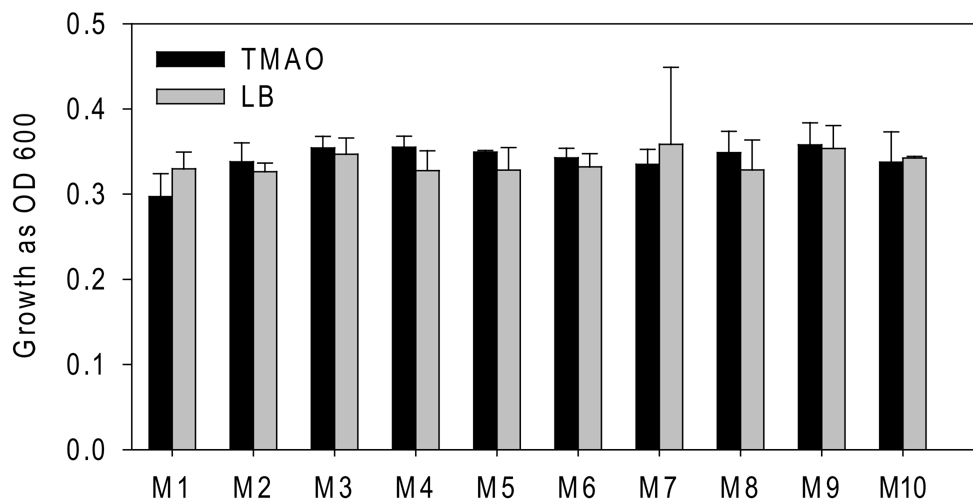
Wild-type Gm-R transformed DH5a cells were grown in medium containing 0.1% Arabinose and Gentamicin in presence or absence of TMAO. Growth was performed in microwell plate incubated at 37°C without shaking. The growth was measured as absorbace at 600nm. Data plotted is normalized with respect to growth without TMAO.

Figure 14



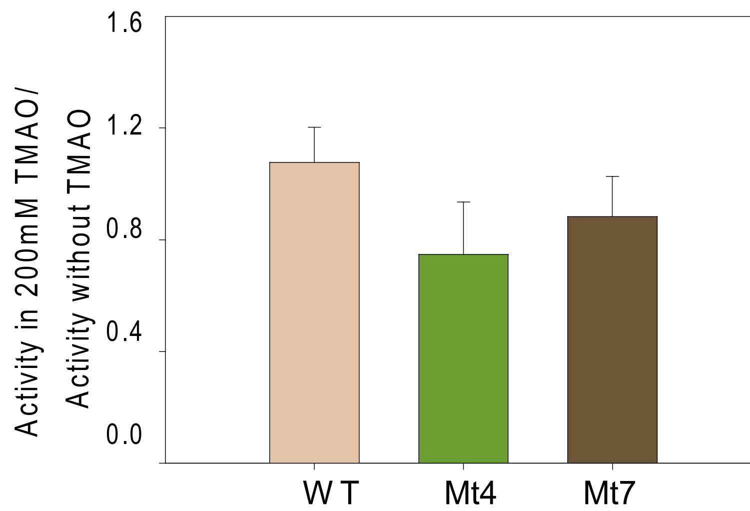
E. coli cells transformed with WtGmR or an inactive GmR was grown at six different concentrations of Gentamicin ranging from 2ug/ml to 134ug/ml, and in presence of 0.1% Arabinose. Growth is measured by OD_{600} and is plotted as a colormap. Cells transformed with WtGmR is able to grow indentically in presence and absence of TMAO. Inactive mutant does not support growth above 2ug/ml of gentamicin both in presence and absence of TMAO. This indicates that TMAO does not reduce the efficacy of gentamicin.

Figure 15



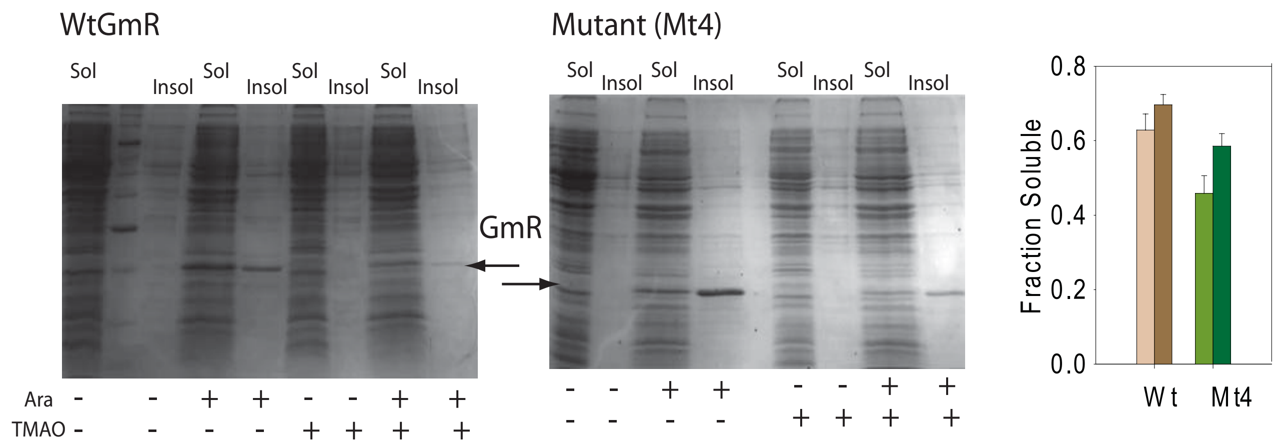
E. coli cells transformed with the mutant plasmids were grown in presence of 0.1% of arabinose and 1mg/ml of gentamicin, a concentration that was found to be insufficient to arrest the growth of non-transformed cells. OD600 of the cultures are plotted after overnight growth in presence and absence of TMAO.

Figure 16



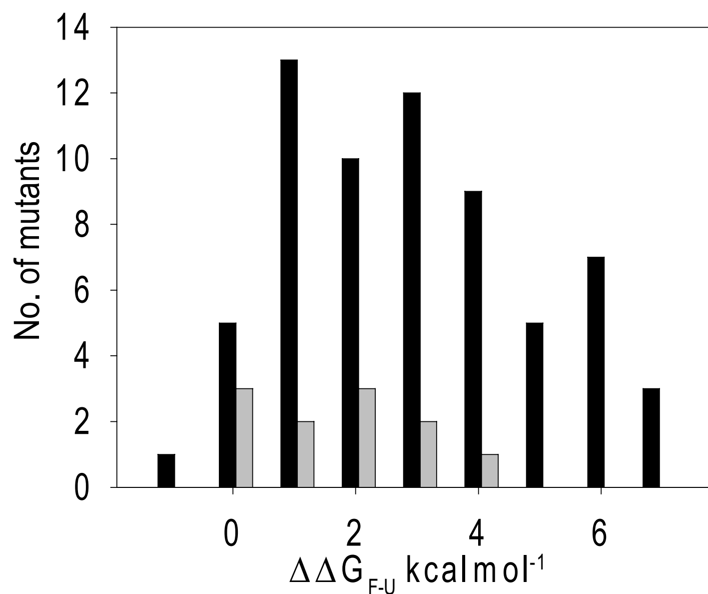
Specific activity of mutants with TMAO.
Activity in presence of 200mM TMAO for 100nM solution of purified proteins (wtGmR, Mt1 and Mt2) is normalized with respect to the activity obtained in absence of TMAO and plotted.

Figure 17



Solubility of WtGmR and Mt4 in presence and absence of TMAO in vivo. Quantitation of the amount of protein was done using ImageJ software.

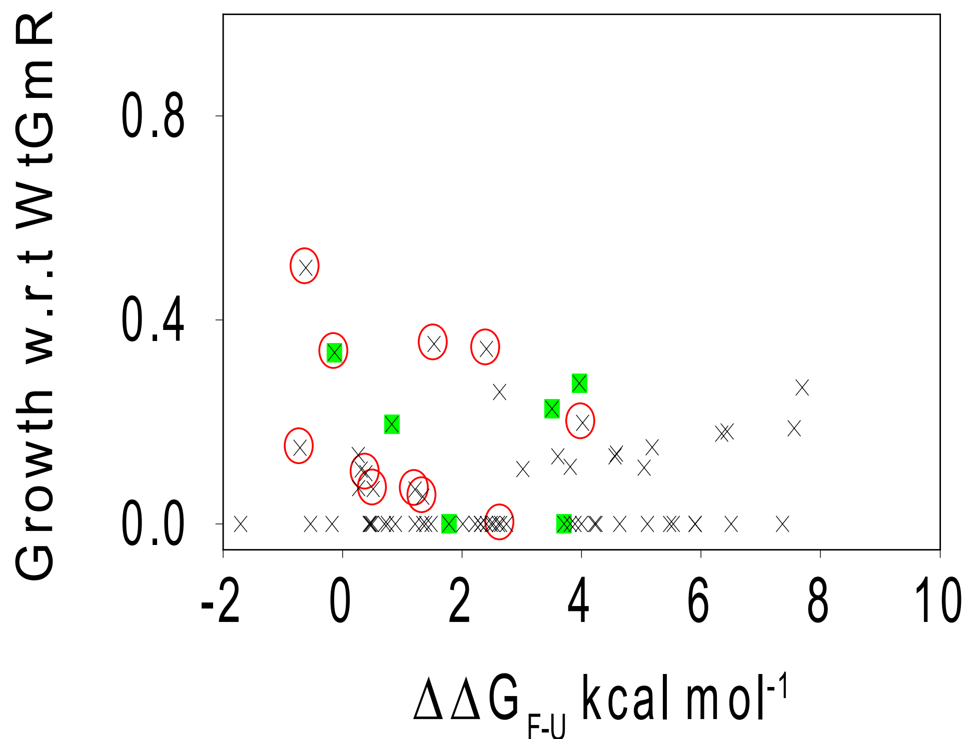
Figure 18



$\Delta\Delta G$ values were calculated for all the GG-mutants using FoldX. Histogram of the $\Delta\Delta G$ distribution is plotted (black bars). Majority of the mutations are destabilizing, with the plot showing non-gaussian distribution. TMAO-buffered mutations, shown in grey bars, are distributed in the lower range of $\Delta\Delta G$ values. The values for $\Delta\Delta G_{FU}$ were calculated as

$\Delta\Delta G_{FU} = \Delta\Delta G_{FU}$ (mutant)- $\Delta\Delta G_{FU}$ (Wild type)

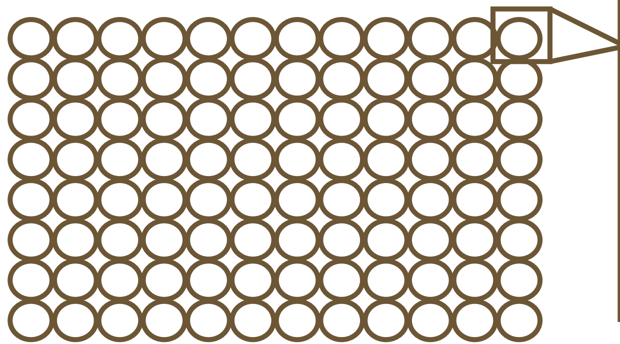
Figure 19

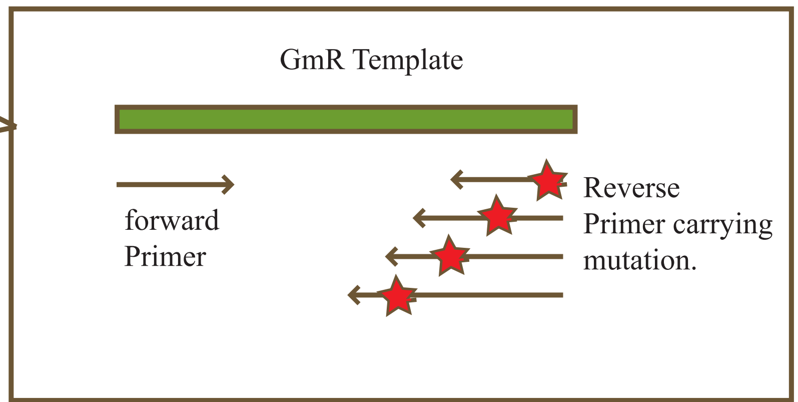


Activity of the different GG-mutants are plotted against the predicted $\Delta\Delta G_{FU}$ values from FoldX due to the corresponding mutations. Mutants buffered by TMAO are circled in red and the mutations rendered active at lower temperature are boxed with green. Though the preponderance of active protein is slightly more in the low $\Delta\Delta G$ region compared to the high $\Delta\Delta G$ regions, there is no statistically significant correlation between the two values.

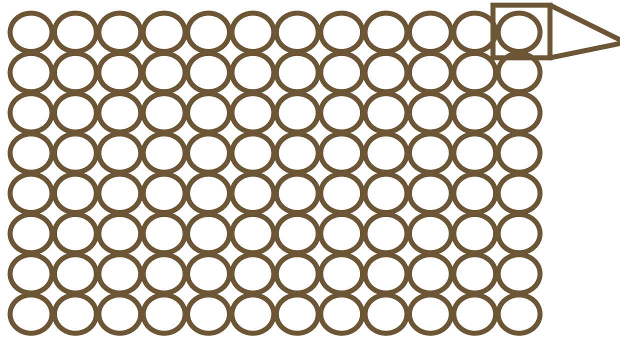
Figure 20

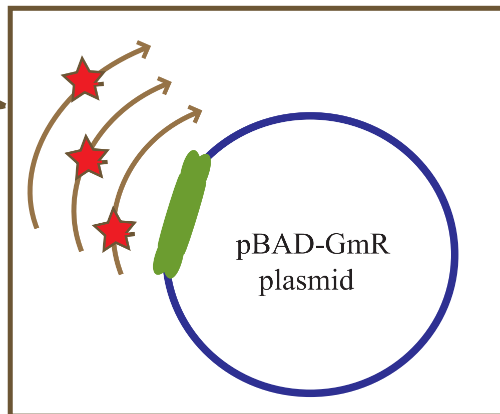
Short PCR



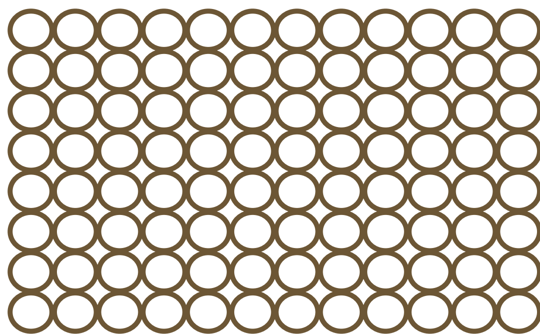


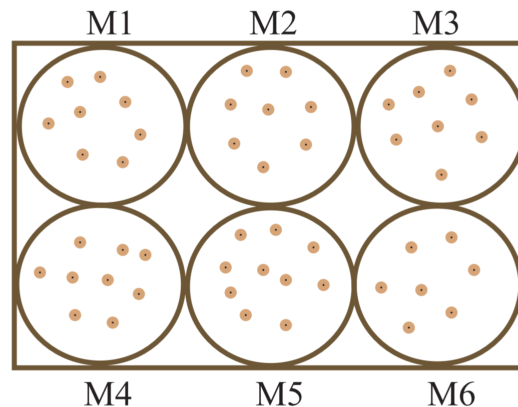
Long PCR





Transformation into E.coli DH5alpha to obtain mutant colonies

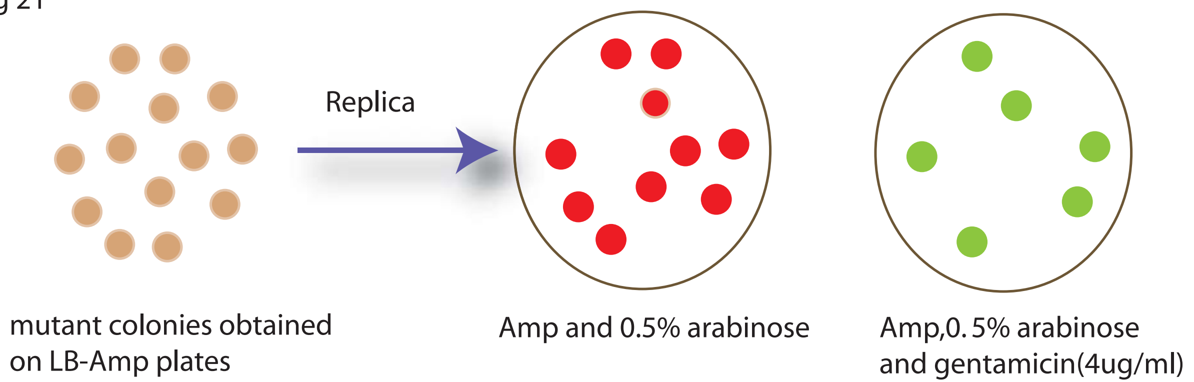


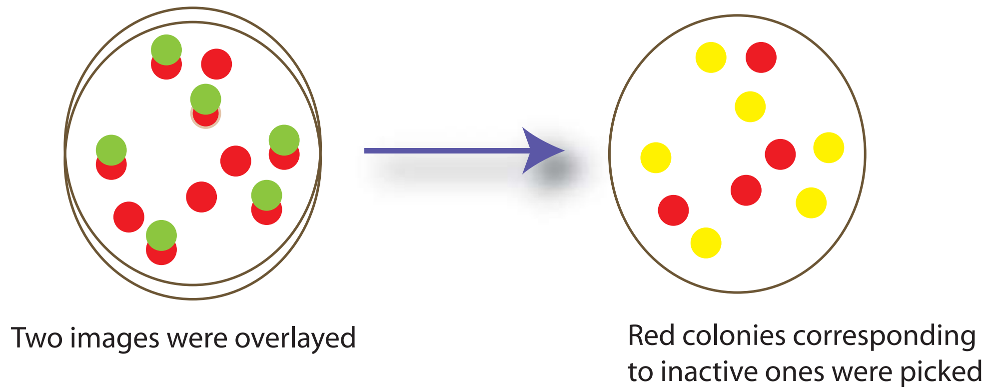


Mutant colonies were scraped into LB and pooled followed by plasmid preparation to obtain pool of mutant plasmids

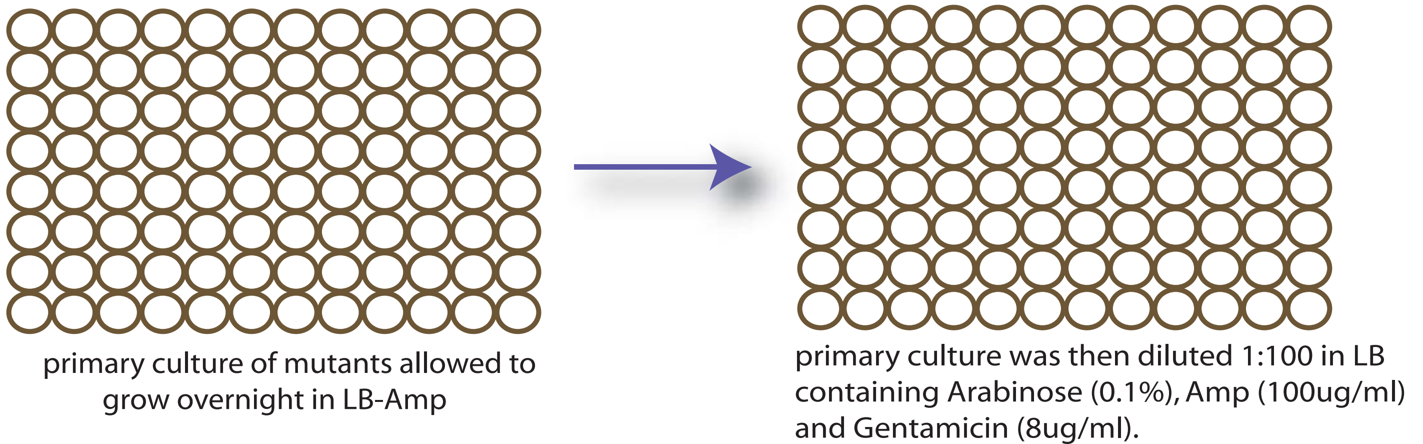
Schematic for generating single-site random mutant library for GmR. Primers containing NNK substitution for each codon was obtained in 96-well format. Individual short PCR was carried out using a constant forward primer and the randmozed reverse primer. The reverse primers contain MNN substitution to encode NNK substitution in the gene. The long-PCR was carried out individually to amplify the whole plasmid and were digested with DpnI subsequently to remove wildtype plasmid. These were then transformed in cells to obtain mutant colonies. Same set of reactions were performed without primers as a control. The control reaction typically yeilded less than 5% of the colonies obtained in the mutant plates. The mutant colonies for each position were pooled together and grown to obtain pooled plasmid representing mutants throughout the gene.

Fig 21



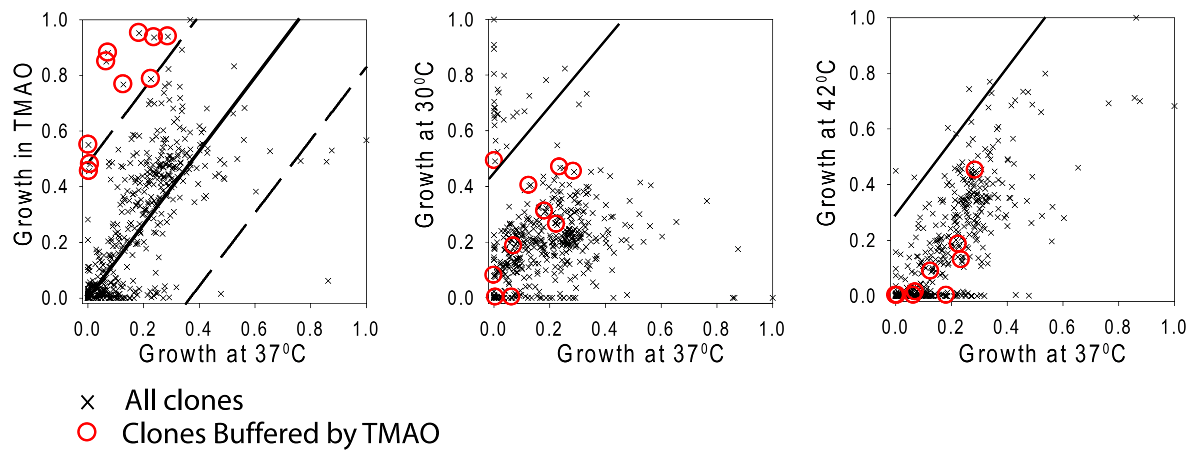


763 GmR Inactive mutants were picked into LB Broth in 96-well format out of which 500 mutants were screened for TMAO dependence.



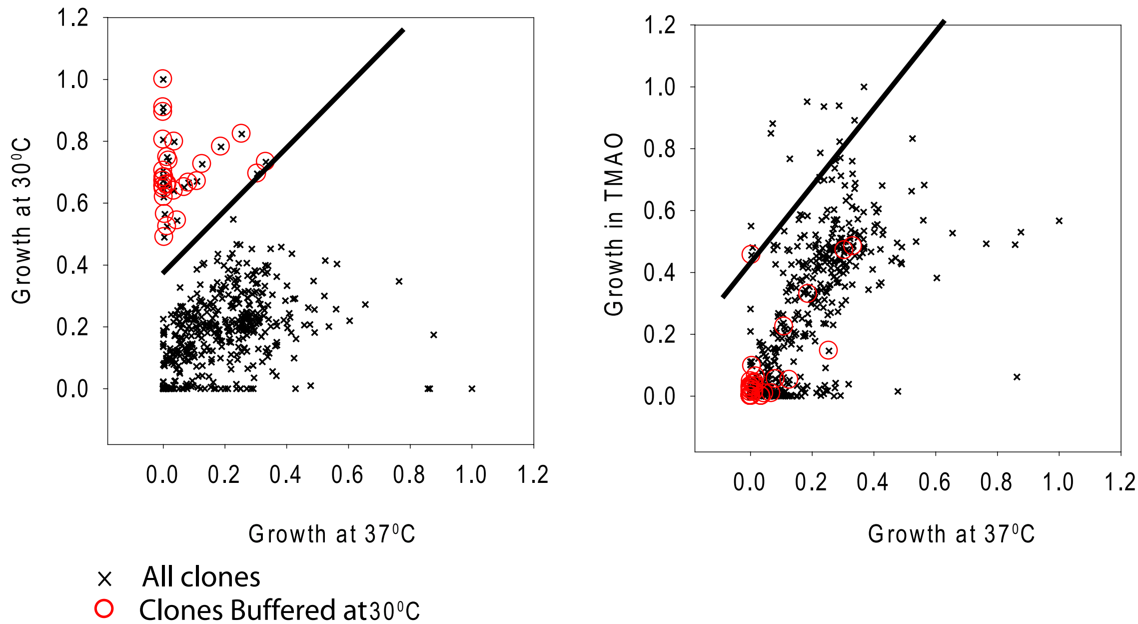
Clones were plated onto LB-amp without gentamicin selection. Following growth the colonies were replica plated onto two plates, one containing gentamicin and one without. Plates were photographed and image analysis tools were used to identify colonies that were growing better in the absence of gentamicin. The selected clonies were picked from the plate without gentamicin selection and inoculated in LB-Amp for further growth.

Fig 22



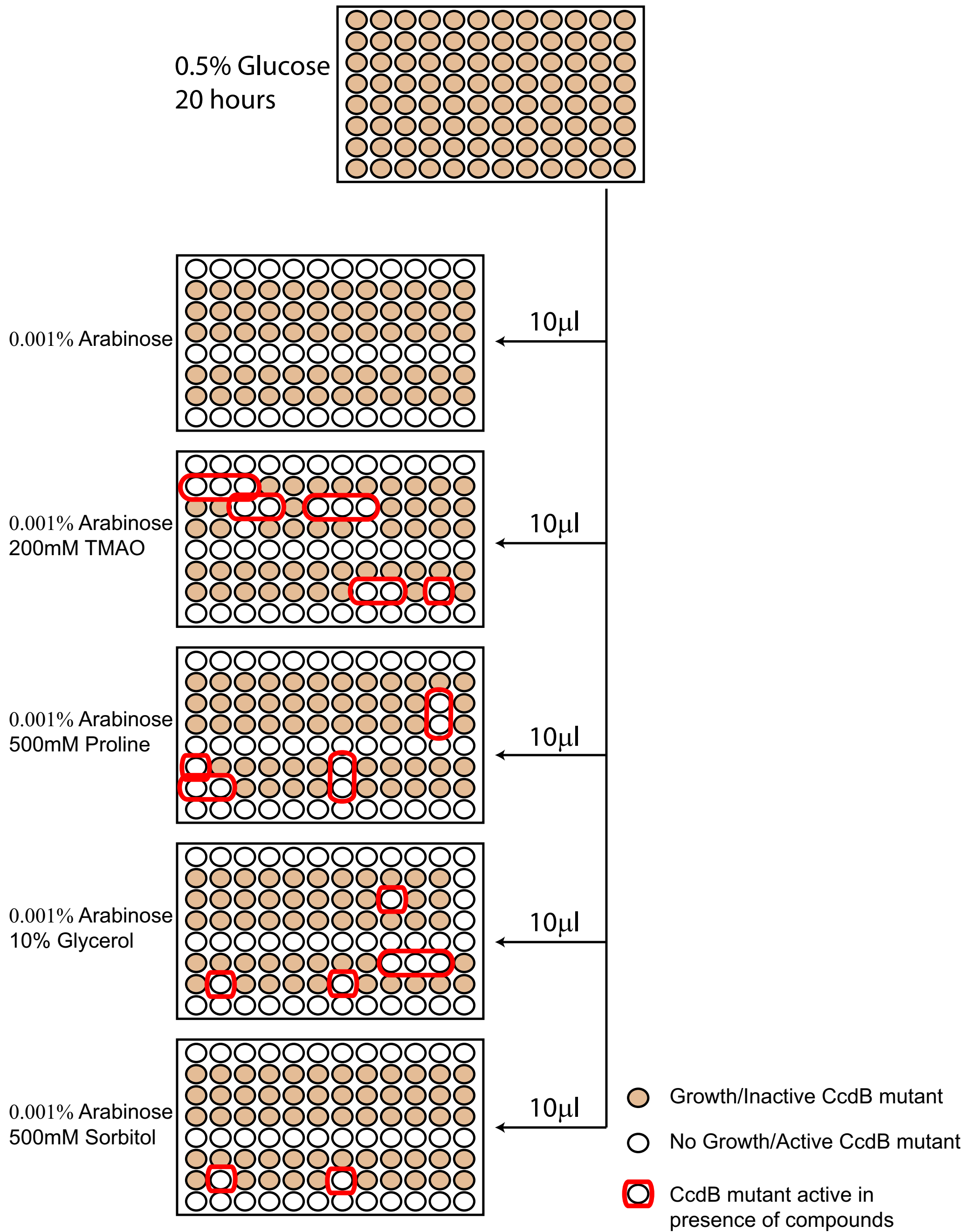
Growth of different mutant clones are plotted to obtain differential growth pattern. Left Panel: Growth in TMAO is plotted against growth in LB to obtain the mutants that grew significantly better in presence of TMAO than in its absence. This was obtained by obtaining a 95% prediction interval around a linear regression through the scatter. Clones falling above the 95% prediction interval were taken to be buffered by TMAO and are circled in red. Middle panel: Growth comparison of clones at 30^{0}C vs normal growth. The clones that showed TMAO buffering are circled in red. Only one clone exhibit better growth at low temperature than at normal temperatur. Right Panel: Similar analysis for growth of clones at 42^{0}C vs normal growth. TMAO-buffered mutants are not buffered at elevated temperatures.

Fig 23



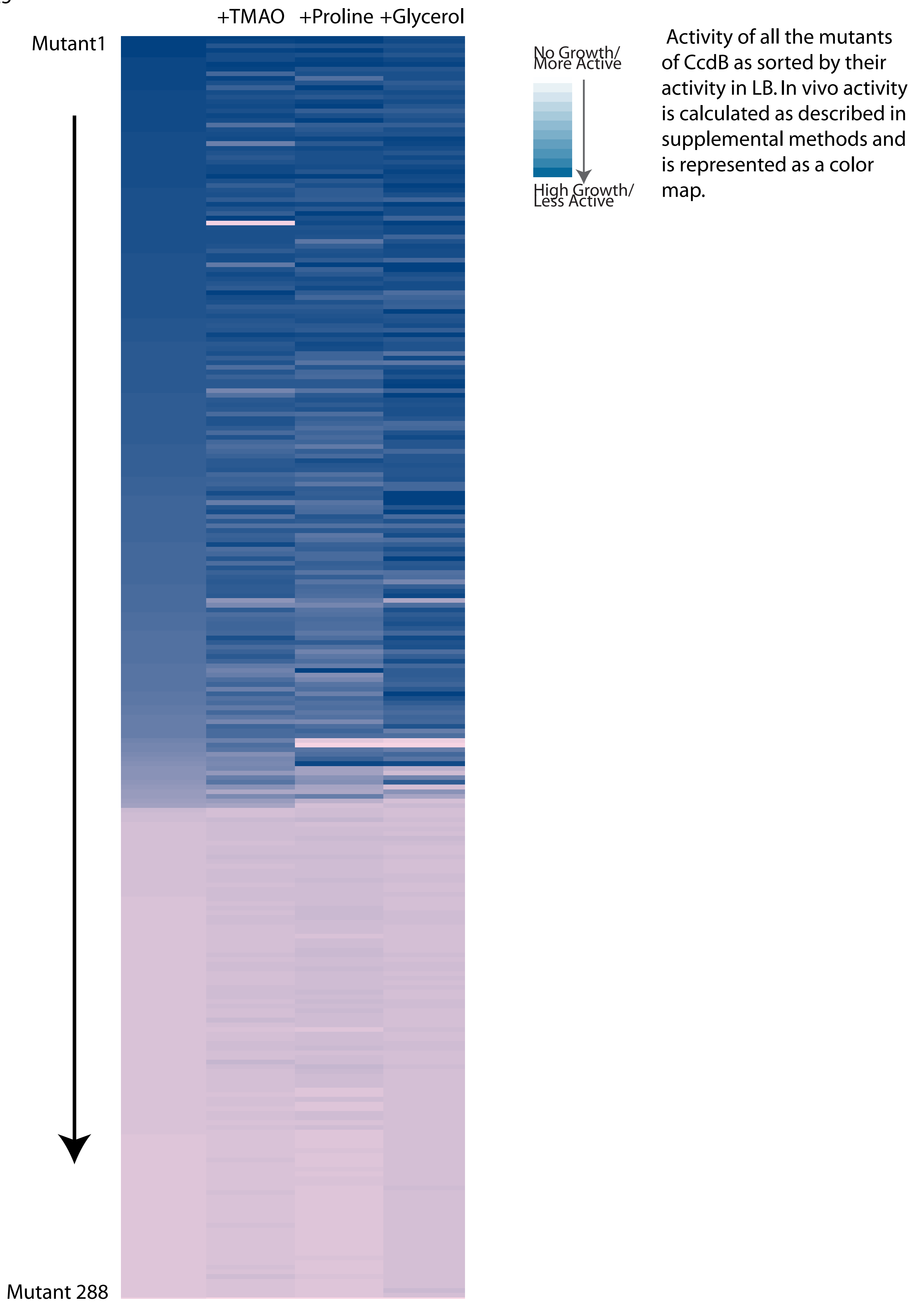
Growth of different mutant clones are plotted to obtain differential growth pattern. Left Panel: Growth comparison of clones at 30°C vs normal growth. The clones that showed temperature sensitive behaviour are circled in red. This was obtained by obtaining a 95% prediction interval around a linear regression through the scatter. Clones falling above the 95% upper prediction interval were taken to be buffered by low temperature and are circled in red. Right Panel: Similar analysis for growth of clones in presence of TMAO vs normal growth. Mutants buffered by low temperature are circled red. One of these mutants show better growth in TMAO than in its absence. Thus temperature sensitive mutants and TMAO-dependent mutants form non-identical sets.

Figure 24



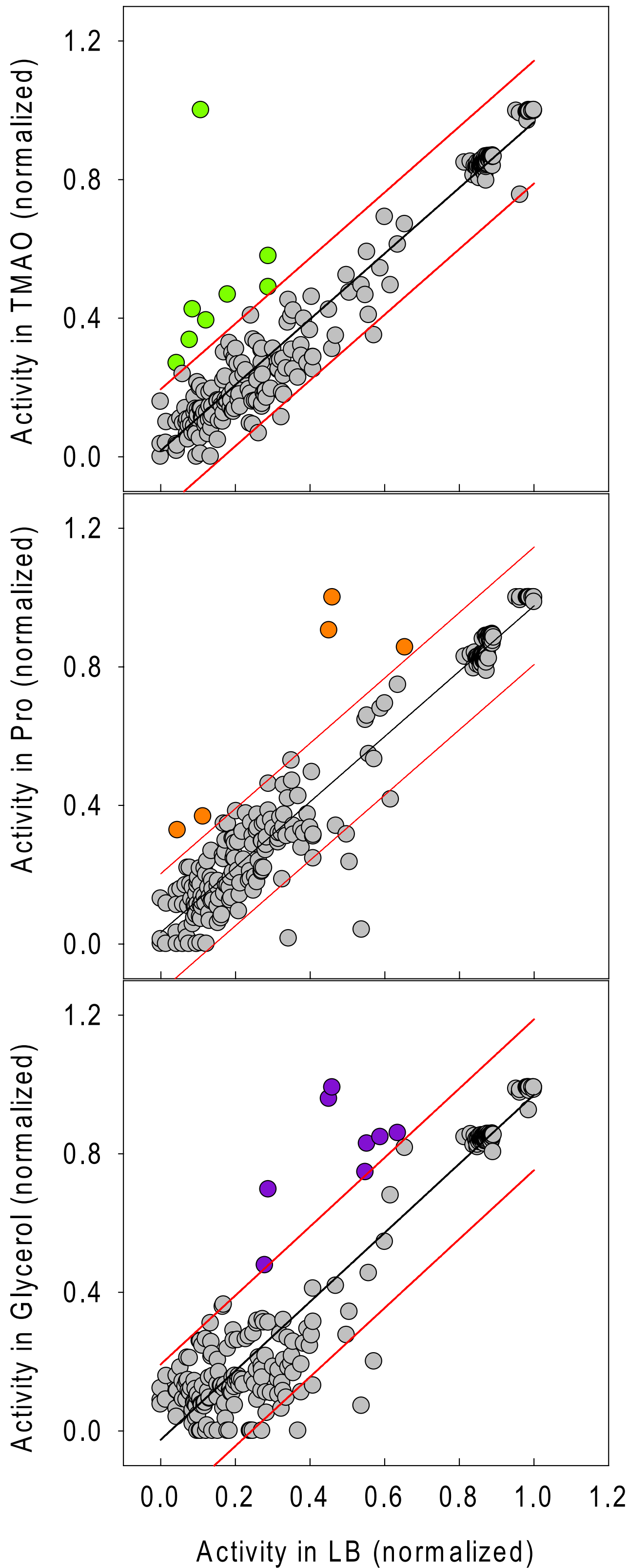
Schematic for the characterization of CcdB activity: CcdB mutants were grown at 37°C in the presence of 0.5% Glucose, to repress the expression from Ara promoter, for 20 hours. 10μl of this culture was inoculated in LB containing 0.001% Ara to induce low level expression of CcdB containing the different osmolytes. Decrease in OD_{600} indicates increased activity of CcdB mutant in presence of specific osmolytes.

Figure 25



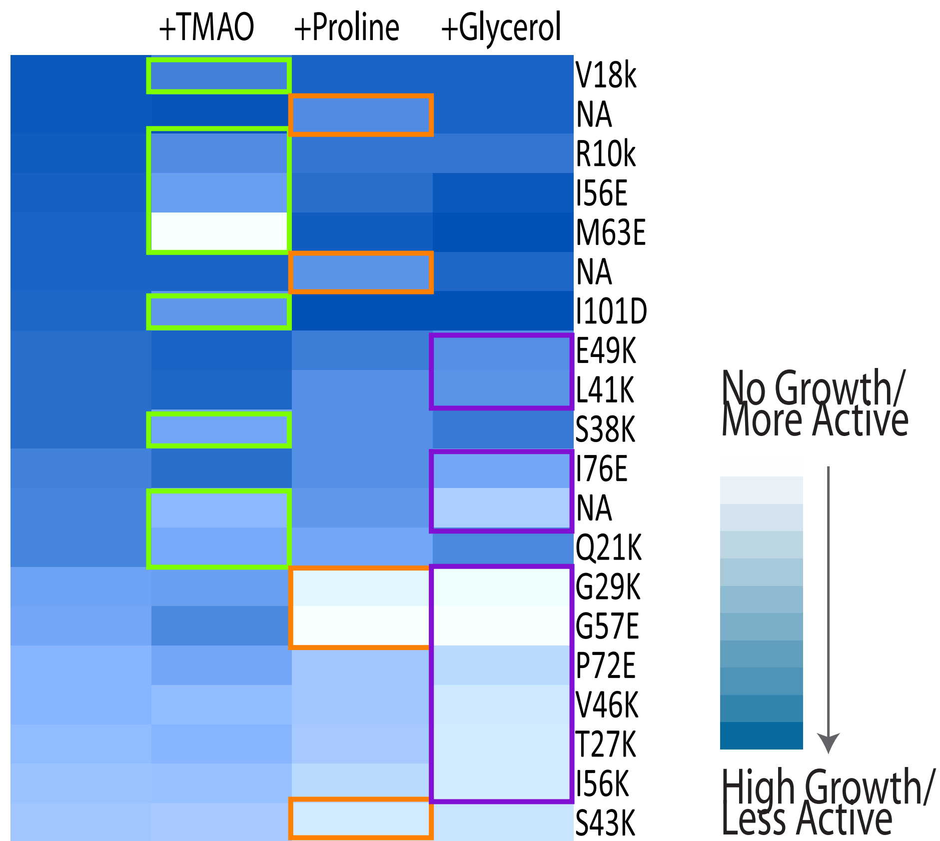
Activity of all the mutants of CcdB as sorted by their activity in LB. In vivo activity is calculated as described in supplemental methods and is represented as a color map.

Figure 26



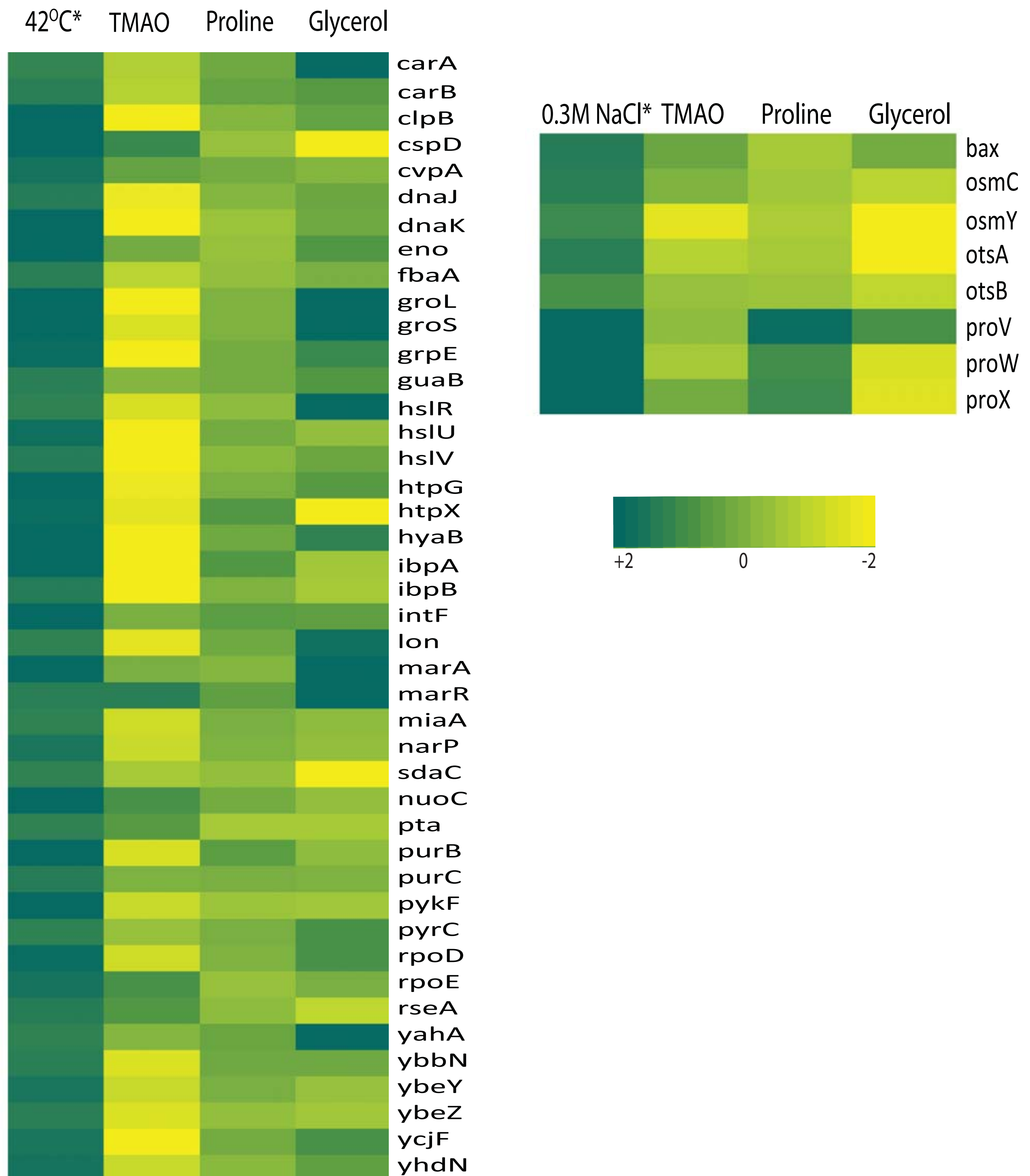
Activity of CcdB was estimated from the decrease afforded by each mutant from the growth of a strain transfomed with an inactive mutant. Activity in presence of the osmolytes were plotted against activity in absence of osmolytes. A linear regression was perfomed (dark line) and the activities seem to correlate quite well in presence or absence of osmolytes. 95% prediction interval was obtained from the regression (red lines) and the clones showing positive deviation from the upper prediction cutoff were designated as the mutations that are buffered. The top panel shows in green the mutants that are buffered in TMAO, in the middle panel, the mutants that are buffered in Proline are shown in orange and in the bottom panel, the mutants that are buffered by glycerol is shown in magenta.

Figure 27



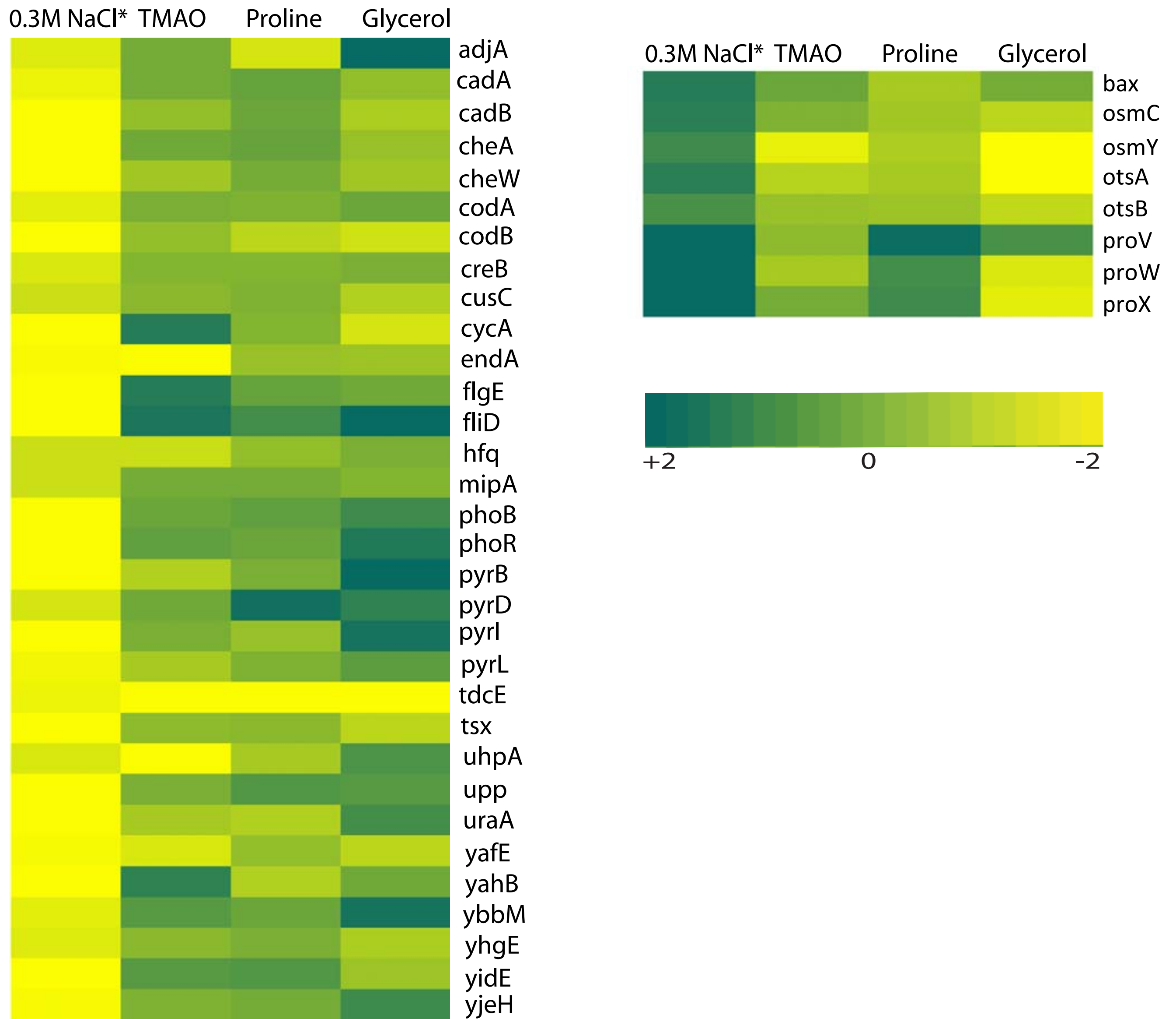
Color map for the growth of CcdB mutants when grown in different conditions. Since CcdB is a toxin, higher growth which is indicated by darker shades of blue, indicates less activity, whereas white indicates a fully active clone.

Figure 28



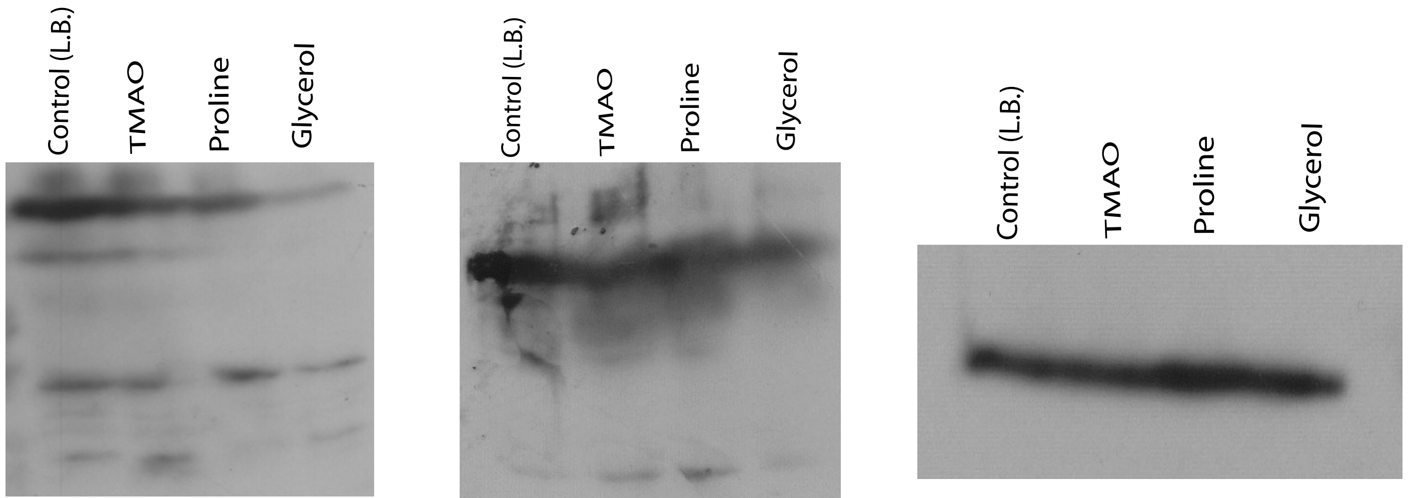
Stress response pathways do not mediate osmolyte mediated buffering. Microarray analysis showing the alterations in E. coli gene expression in presence of different osmolytes. The genes found to be upregulated in heat shock and osmotic shock were obtained from the supplemental materials of Gunasekera et al 2008 [2] and were plotted here along with their fold change values to compare these expression profiles. Genes upregulated upon Heat Shock (Left Panel) and Osmotic Shock (Right Panel) were are compared with their expression values in presence of the different osmolytes.

Fig 29



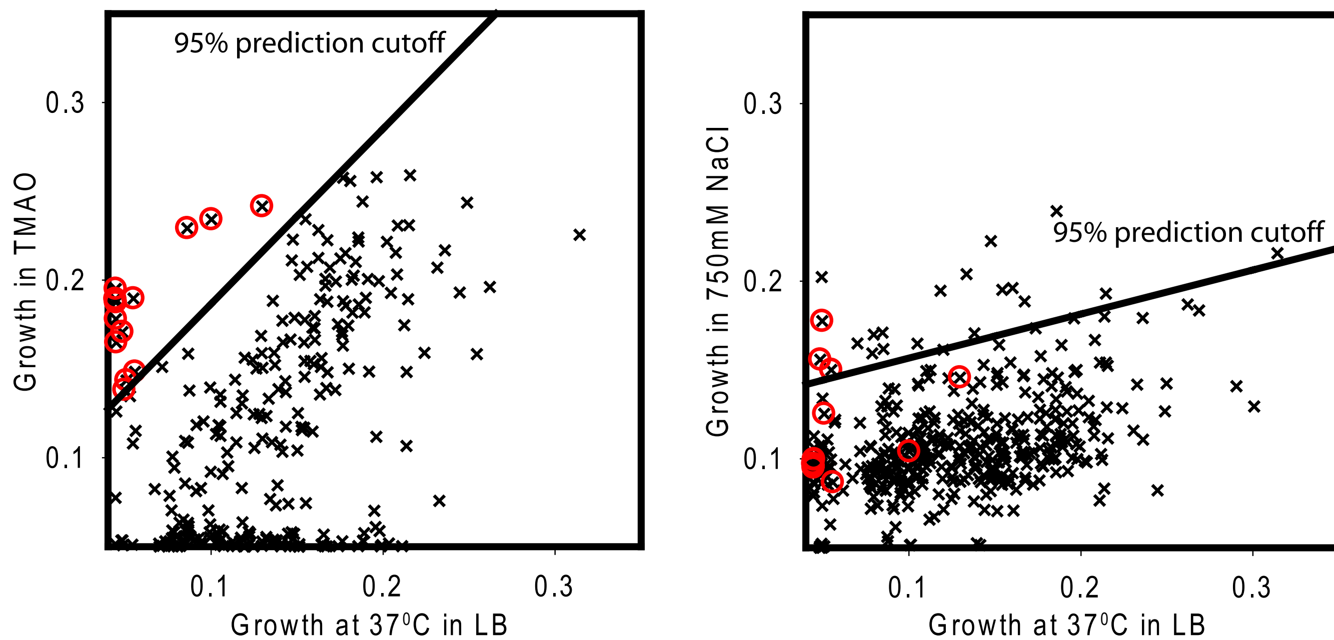
Genes that are downregulated in Heat shock (lef panel) and (right panel) osmotic shock are plotted alongwith their fold change values to compare their expression profiles in presence of different osmolytes.

Fig 30



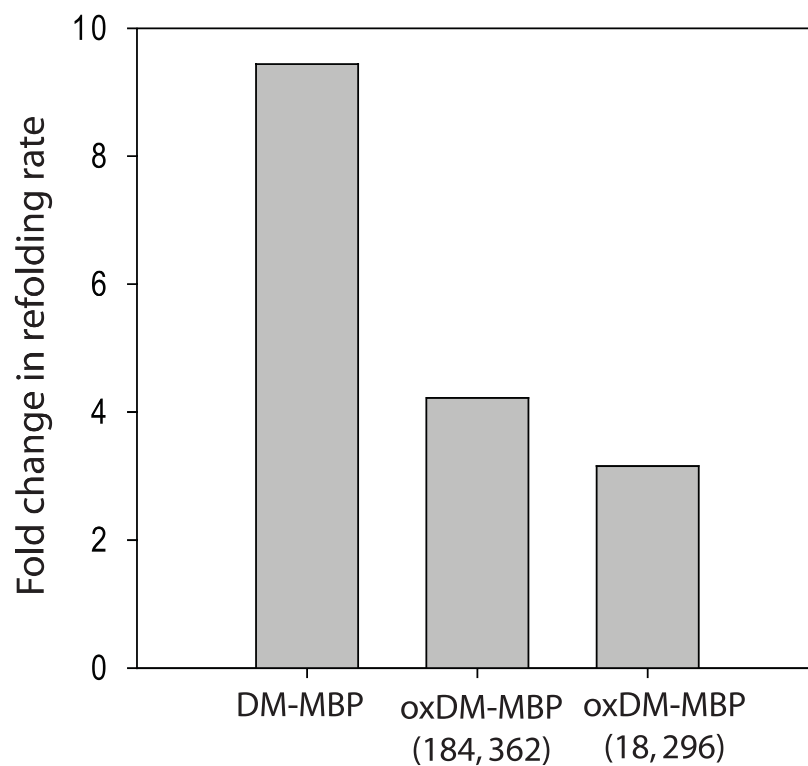
osmolytes do not alter the protein levels of Molecular Chaperones. Expression level of DnaK(left panel), GroEL (middle panel) and trigger factor (right panel) was measured using western-blotting.

Figure 31



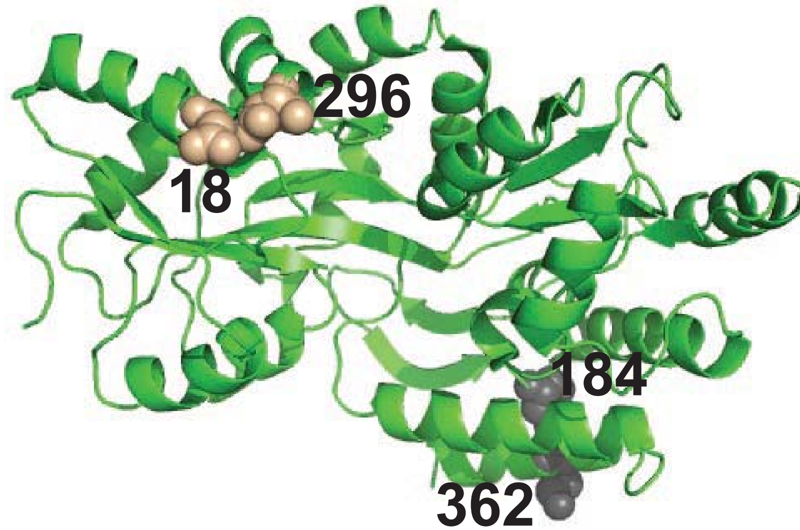
Gm-R random mutants buffered under osmotic shock conditions are distinct from TMAO dependent ones : Growth for 500 mutant clones of Gm-R in presence of the osmolytes or at high salt concentration is plotted against normal growth in LB. 95% prediction interval was obtained from the regression (black line) and the clones showing positive deviation from the upper prediction cutoff were designated as the mutations that are buffered. Left Panel: In red are circled the clones that are buffered in presence of TMAO. Right Panel: The same clones are circled to show the activity of these clones in presence of elevated salt. Only three of the ten TMAO buffered clones show buffering in presence of hyperosmotic stress.

Fig 32



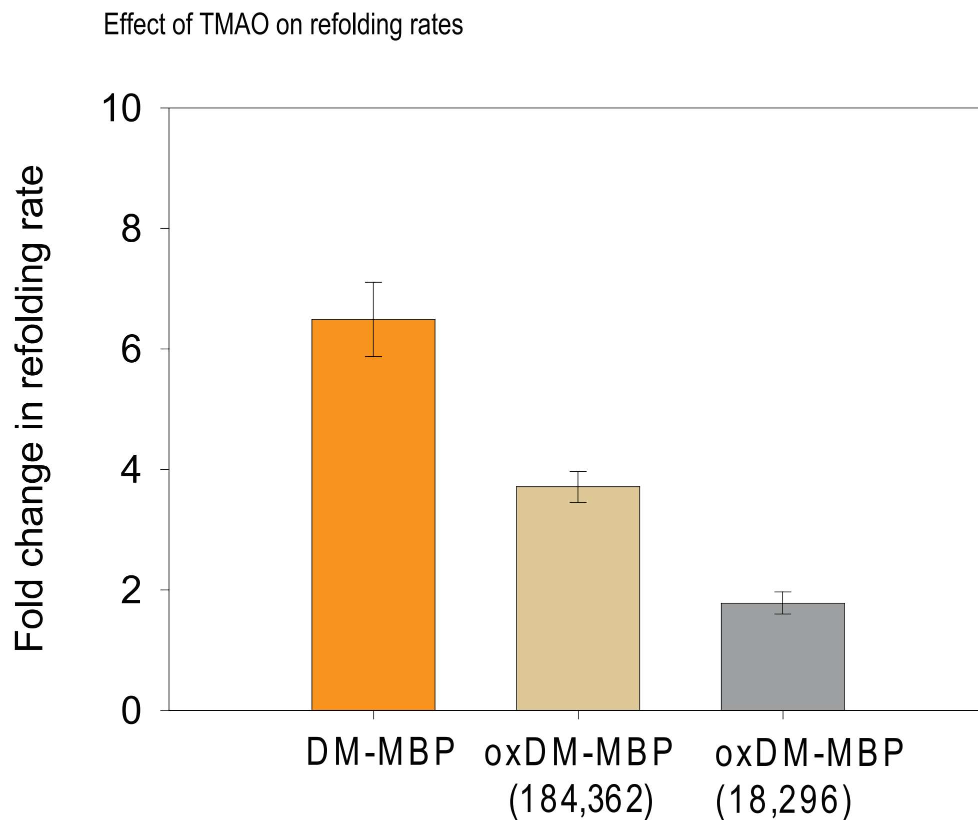
Refolding of DM-MBP and its variants were performed in vitro as explained previously. In this case 500mM Trehalose was present in the refolding buffer to examine the effect of trehalose on refolding activity. Fold change was obtained by dividing the rate obtained upon folding in presence of trehalose by the rate obtained upon refolding in the absence of trehalose.

Figure 33



Crystal structure of MBP(1omp) indicating the residues substituted with cysteines to obtain two disulfide variants of DM-MBP; 18 and 296 (beige), 184 and 362 (grey).

Figure 34



Folding rates of DM-MBP, oxDM-MBP(18,296) and oxDM-MBP(182,362) were obtained in presence and absence of TMAO. Rates obtained in presence of 0.5 M TMAO were normalized with respect to the rate obtained in absence of TMAO to obtain the fold change in refolding rate in presence of 0.5 M TMAO. Refolding reactions were carried out as described previously (Fig.1h).