

A green fluorescent protein screen for identification of well-expressed membrane proteins from a cohort of extremophilic organisms

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Abstract: Green fluorescent protein (GFP) fusion proteins provide a potentially facile tool for identification of well expressed, properly behaved membrane proteins for biochemical and structural study. Here, we present a GFP-expression survey of >300 membrane proteins from 18 bacterial and archaeal extremophiles, organisms expected to be rich sources of membrane proteins having robust biophysical properties. We find that GFP-fusion fluorescence intensity is an excellent indicator of over-expression potential. By employing a follow-up optimization protocol using a suite of non-GFP constructs and different expression temperatures, we obtain 0.5–15 mg L⁻¹ expression levels for 90% of the tested candidate proteins that pass the GFP screen. Evaluation of the results suggests that certain organisms may serve as better sources of well-expressed membrane proteins than others, that the degree to which codon usage matches the expression host is uncorrelated with success rate, and that the combination of GFP screening and expression optimization is essential for producing biochemically tractable quantities of material.

Keywords: membrane protein expression; GFP-screen; extremophile

Introduction

Membrane proteins are the gateways for signals that pass between a cell and its surroundings and are central to ionic signaling mechanisms and the material transport required for nutrient uptake and toxin dis-

posal. Understanding membrane protein molecular architecture remains one of the major challenges of modern structural biology efforts. One of the main obstacles is the difficulty associated with obtaining sufficient quantities of pure material for biochemical and structural analysis.^{1,2} Because most membrane proteins are not found in readily accessible over-enriched native sources, it is necessary to develop ways to overproduce membrane protein constructs at will. The ability to overproduce proteins in heterologous systems has revolutionized structural biology studies of soluble proteins but similar scale successes have not yet been had for membrane proteins. Thus, pursuit of robust methods to express and characterize membrane proteins remains an area of vigorous research efforts.

The most rigorous test of whether a protein can be crystallized is to overexpress, purify, and place the

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protein of interest in crystallization trials. However, taking any given protein through this procedure requires a large investment of time and resources and has a high probability of a negative outcome for any given candidate. There have been ongoing efforts to develop methods that serve as readily accessible proxies to identify well-expressed, well-behaved protein samples that should have a higher probability of leading to crystallizable material without the need for an initial complete characterization of each candidate. Such approaches attempt to leverage the power of molecular biology to provide a rapid way to screen large numbers of constructs in parallel and identify the most promising for further, more intensive characterization. A leading tactic in this regard has been the use of fusion proteins bearing Green Fluorescent Protein (GFP).^{3–9} Because of its spectral characteristics, GFP fluorescence provides a unique and sensitive means for detecting tagged proteins within a cellular environment. Maturation of the GFP chromophore and development of the unique GFP spectral signal are directly coupled to proper GFP folding.^{10,11} Because of this property, there should be a correlation between the fate of the target protein and the fate of the GFP chromophore.³ Thus, the level of fluorescence signal from a fused GFP molecule is expected to provide a reasonable measure of the ability of the candidate protein to be expressed at high levels in a properly folded form.

Here, we report the implementation of a *E. coli*-based whole cell, GFP fluorescence assay to survey the expression properties of a large cohort of membrane proteins (314 candidates) from extremophilic organisms. We show that measurement of the fluorescence produced by a C-terminal GFP fusion of the candidate protein relative to a set of benchmark GFP-fusion proteins provides a reliable way to identify membrane proteins that can be expressed in the 0.5–15 mg L⁻¹ range in the membrane fraction following expression optimization. The production of such quantities facilitates further characterization by detergent extraction screens and size exclusion chromatography. Importantly, our results demonstrate that the use of the whole-cell fluorescence detection method provides a rapid way to assess the overexpression potential of many membrane protein with a limited investment of labor. This stands in sharp contrast to traditional, labor-intensive methods for expression screening using SDS-PAGE and Western blot. Analysis of candidate protein expression behavior with respect to protein properties suggests that although there appear to be no particular limits on protein size or number of transmembrane segments, proteins in the 20–50 kDa range and having <7 transmembrane segments have a higher success rate. Interestingly, we find no correlation between expression level and the degree to which the codon usage of the target gene matches the expression host.

Results

Fluorescence-based screening for identifying membrane proteins having overexpression potential

Following previous reports using GFP as a reporter for membrane protein expression,^{4,7–9,12} we established an *E. coli* GFP-based expression system (Supporting Fig. 1) to evaluate the expression potential of a large cohort of membrane proteins from organisms found in extreme and unusual environments. To allow rapid clone generation, we constructed a ligase independent cloning vector¹³ to facilitate direct cloning of PCR products of the gene of interest (Supporting Fig. 1). Target protein fusions contained a C-terminal tag that bore in sequence, a Tobacco Etch Virus (TEV) protease site,¹⁴ eGFP,¹⁵ and an octahistidine (His₈) tag [Fig. 1(a)]. On the basis of previous studies,^{4,7–9,12} we anticipated that there was likely to be a correlation between proper folding and membrane insertion of the target protein and GFP fluorescence. One limit to this assay is that the C-terminal GFP moiety will only mature if the C-terminus of the target protein is intracellular.⁷ As a consequence, any well-expressed membrane proteins having a topology that places the C-terminus extracellularly will be missed. An estimated 70% of integral membrane proteins have their C-terminus in the cytoplasm.¹⁶ Given the expected high proportion of candidates with cytosolic C-termini, and the possibility that a single error in transmembrane protein prediction would eliminate potential candidates by inadvertently switching the predicted topology, we did not use topology prediction algorithms to prescreen candidates.

We chose candidate membrane proteins from organisms that live in extreme pH, high salt, the presence of high concentrations of heavy metals, high temperatures, or some combination of these extremes, based on the rationale that proteins found in such organisms, and in particular those in the membrane, which should have direct contact with the harsh environment, are likely to be very stable and robust subjects for biochemical and crystallographic studies.¹⁷ The criteria for selection of candidate organisms were that in addition to living in an unusual environment the selected organisms had to have a completely sequenced genome and have genomic DNA that could be obtained from the ATCC collection to be used as template for PCR.

Membrane proteins were selected for the expression survey using a combination of annotation searches for ion channel, transporter, exchanger, antiporter, carrier, and major facilitator superfamily members, and by searching the genome of each candidate organism with a generic transmembrane helix profile. From an initial list of ~400 selected candidates, we succeeded in making 314 GFP fusion constructs and tested their expression levels by measuring in cell GFP

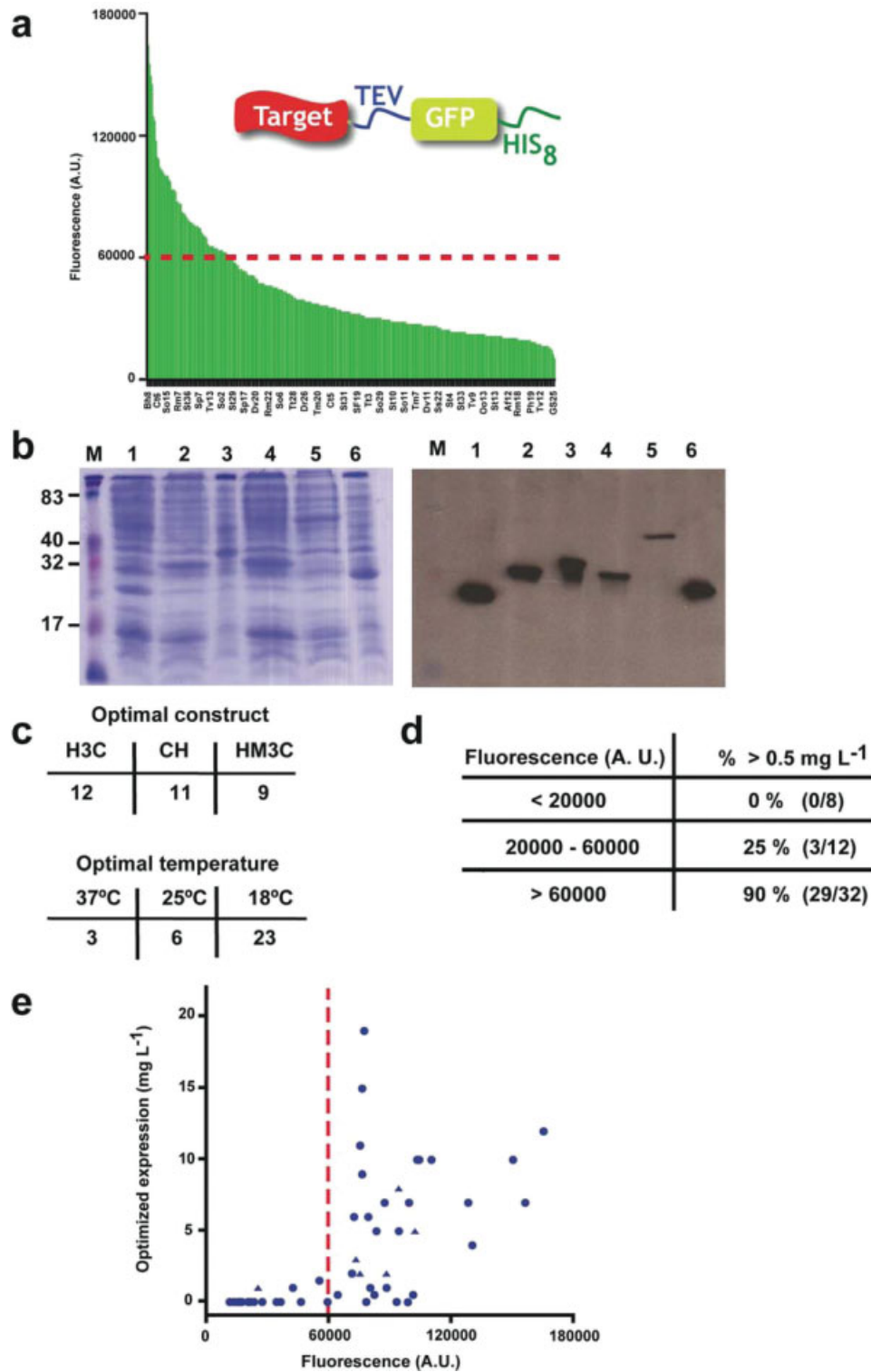


Figure 1. GFP-based integral membrane protein expression screen. **(a)** Fluorescence measurements of 314 GFP fusions assayed in *E. coli*. Dashed line indicates the cutoff threshold level based on the values obtained three benchmark proteins, KvAP, AQPz, and YhjX. Inset shows schematic of GFP constructs. **(b)** SDS-PAGE analysis of the C41 *E. coli* membrane fractions from several extremophile membrane protein candidates following expression optimization analyzed by coomassie-blue staining (left) and anti-polyhistidine tag western blot (right). Lanes are: M, Marker; 1, Af27 (22 kDa); 2, Rm29 (30 kDa); 3, Rm8 (30 kDa); 4, Ph11 (29 kDa); 5, Sp2 (49 kDa); 6, Rm4 (25 kDa). **(c)** Tables showing the numbers of proteins having optimal expression as H3C, CH, and HM3C affinity tagged constructs and showing the numbers of proteins having the optimal expression at different expression temperatures. **(d)** Percentage of optimized candidates with expression >0.5 mg L⁻¹ of culture, grouped according to their fluorescence values from the GFP screen. **(e)** Comparison of GFP fluorescence versus final amount of protein following optimization. Expression levels were determined by following single-step metal affinity purification in FC-12 and measurement of the amount of protein purified by absorbance (circles) or by comparison with a protein standard by western blot (triangles).

Table I. List of Extremophilic Organisms Selected, Number of Candidates Screened, Number Positive, and Hit Rates in Each Organism

Organism	Environment	Candidates screened	>60,000 F.A.U.	
			# Positive	% Positive
<i>Aeropyrum pernix</i>	Hydrothermal vent	8	1	13
<i>Archaeoglobus fulgidus</i>	Hydrothermal vent, oil deposits, hot springs	21	7	33
<i>Bacillus halodurans</i>	pH >9.5, high salt, Dead Sea	5	1	20
<i>Chlorobium tepidium</i>	New Zealand hot spring	6	3	50
<i>Deinococcus radiodurans</i>	Radiation resistant	14	3	21
<i>Desulfovibrio vulgaris</i>	Tolerates heavy metals	24	3	13
<i>Geobacter sulfurreducens</i>	Hydrocarbon contaminated soils, tolerates heavy metals	13	2	15
<i>Oenococcus oeni</i>	Low pH, high alcohol	17	1	6
<i>Pyrococcus furiosus</i>	Geothermal marine sediments	6	1	17
<i>Pyrococcus horikoshii</i>	Hydrothermal vent	21	3	14
<i>Ralstonia metallidurans</i>	Heavy metal containing industrial wastes	25	11	44
<i>Shewanella oneidensis</i>	Heavy metal reducer	26	8	31
<i>Silicibacter pomeroyi</i>	Sea water, sulfur reducer	12	6	50
<i>Sulfolobus solfataricus</i>	Volcanic hot springs	19	0	0
<i>Streptococcus thermophilus</i>	Yoghurt, cheese, low pH	30	7	23
<i>Thermotoga maritima</i>	Geothermal marine sediments	20	3	15
<i>Thermus thermophilus</i>	Yellowstone hot spring	18	3	17
<i>Thermoplasma volcanium</i>	Hot springs, low pH	29	1	3
Total		314	64	20

fluorescence following induction and expression at 25°C (Supporting Table I).

Comparison of expression using a simple fluorescence assay that measured the GFP fluorescence signal from a standardized density of cells of the 314 GFP fusions yielded a wide distribution of fluorescence values, 20,000–180,000 F.A.U. (Fluorescence Arbitrary Units) [Fig. 1(a), Supporting Table I]. Uninduced cells grown to similar density yielded a background value of ~20,000 F.A.U. To set a criterion for the level of expression that would indicate the most promising candidates, we examined the performance of three benchmark membrane protein-GFP fusions. Two of the benchmark proteins were chosen because they have had X-ray crystallographic structures determined from *E. coli* expressed material, AQPz^{18,19} and KvAP.^{20,21} The third benchmark protein, YhjX, was the best expressed membrane protein in a GFP-based expression survey of *E. coli* membrane proteins.⁶ Our rationale was that expression of any test protein-GFP fusion at levels equivalent to or better than these

benchmarks should indicate the production of acceptable levels of properly folded material for further characterization. On the basis of the benchmark protein-GFP fusion values, AQPz (73,000 F.A.U.), KvAP (118,000 F.A.U.), and YhjX (97,000 F.A.U.), we defined a positive threshold of 60,000 F.A.U. This value was chosen to allow a generous lower bound for candidates that should be as good or better than the benchmark proteins. Sixty-four of the screened candidates (20%) yielded fluorescence values at or above the threshold (60,000 A.U.). Interestingly, we obtained vast differences in success among the extremophilic organisms (Table I). *C. tepidium*, *R. metallidurans*, and *S. pomeroyi* were good sources of well-expressed candidates, having success rates as high as 40–50%, whereas *O. oeni*, *S. solfataricus*, and *T. volcanium* proved to be very poor sources having very low success rates, 0–6%. Further analysis revealed that certain membrane protein classes express better than others (Table II). For example, candidates from the mechanosensitive ion channel and from the

Table II. Expression Success Analyzed by Family

Membrane protein class	Candidates tested	% of total candidates	% of candidates >60,000 F.A.U.	
			>60,000 F.A.U.	>60,000 F.A.U.
Antiporter	25	8.0	3	12.0
Exchanger	12	3.8	2	16.6
Ion channel	24	7.6	6	25.0
MATE transporter	6	1.9	2	33.3
MotA proton channel	7	2.2	5	71.4
Mechanosensitive ion channel	28	8.9	15	53.5
Transporter	177	56.4	27	15.2
Other	35	11.1	4	11.4
Total	314		64	20.3

Analysis of success rate by membrane protein class.

flagellar motor MotA proton channel families had high success rates (53.5% and 71.4%, respectively), whereas candidates from the antiporter, exchanger, and transporter families were less likely to express well (12.0%, 16.6%, and 15.2%, respectively). The origins of the differences are unclear but may relate to a combination of protein molecular weight, total number of transmembrane segments, toxic consequences of protein activity from the overexpressed protein, and the potential requirement for auxiliary subunits that were not included in the expression study.

Overexpression optimization without GFP

Initial attempts at direct purification of a number of GFP constructs having high F.A.U. scores (Af11, Bh8, Ct6, and Dr35) proved problematic and did not directly yield sufficient quantities of purified material for further characterization. In consideration of the possibility that the GFP itself was proving a burden to production of the candidate protein, we cloned 52 candidates from three different pools; 32 high-expressing proteins (>60,000 F.A.U.), 12 medium to low expressing proteins (60,000–20,000 F.A.U.), and 8 low expressing proteins (<20,000 F.A.U.) into three affinity tag formats for protein expression optimization experiments. These 156 constructs were subjected to overexpression optimization trials in which each construct was grown as a small scale culture (7 mL) and tested for expression at three different induction temperatures (18°C, 25°C, and 37°C). Expression levels for each sample were assayed evaluating SDS-PAGE by coomassie blue staining and western blot using an anti-polyhistidine antibody.

The optimization experiments with non-GFP constructs caused clear improvements compared to the temperature-optimized GFP fusion construct of the same target (Supporting Fig. 2). Figure 1(b) shows examples of membrane fractions for six of the candidate proteins following expression optimization. Thirty-two candidates of the 52 showed expression by both coomassie-staining and Western blot analysis. Both the affinity tag and expression temperature were important variables for expression optimization [Fig. 1(c)]. No particular tag predominated as the best choice. In general, a low induction temperature, 18°C, proved most beneficial. Comparison of the amount of protein produced under optimized expression conditions by using single step using metal affinity purification in Fos-choline-12 (FC-12), which was found as the most robust detergent for membrane protein extraction (see below), followed by quantification of the amount of protein expressed in the membrane fraction showed clear differences between the ability to convert proteins from different GFP score categories into expressed material [Fig. 1(d,e)]. Strikingly, we were able to produce 90% of the candidates from the high-expressing category at levels of $\geq 0.5 \text{ mg L}^{-1}$ [Fig. 1(d)] with 50% of these made at $\geq 5 \text{ mg L}^{-1}$. The success

rate for producing $\geq 0.5 \text{ mg L}^{-1}$ of protein from the medium to low expressing category was also good, 25%. None of the candidates from the category having GFP values near the background fluorescence value gave expression levels $> 0.5 \text{ mg L}^{-1}$ following optimization. Comparison of the amounts of expressed proteins following optimization with the GFP screen fluorescence values shows that the fluorescence value served as a reliable indicator of candidate proteins that could be expressed in the membrane fraction at a level that is useful for further biochemical characterization [Fig. 1(e)]. On the basis of these success rates, we estimate that 90% of the candidates that passed the GFP screen could be produced at $\geq 0.5 \text{ mg L}^{-1}$ culture if subjected to optimization experiments. These results highlight the value of including benchmark standards in execution of GFP-based protein expression surveys and indicate that a simple GFP screen should be sufficient for initial identification of proteins with a high-likelihood of overexpression potential.

Detergent screening by size exclusion chromatography

Detergents are a necessary component of any membrane protein purification effort and constitute a complex variable that requires extensive investigation.^{22,23} Detergent choice has significant implications for protein oligomeric state, stability, homogeneity, and crystallization. Unfortunately, it is impossible to predict which detergent will be suitable for extraction, purification, and crystallization of a given membrane protein. Having sufficient expressed material for a variety of candidate proteins from different membrane protein classes following our expression optimization experiments, we screened candidates for detergent solubilization and size exclusion profile behavior using a panel of several different detergents. These experiments were aimed at assessing whether the overexpressed material could yield protein that would be of suitable quality for further biophysical characterization and crystallization trials. We chose 24 well-expressed membrane proteins and used a panel of six commonly used detergents decyl- β -D-maltoside (DM), octyl- β -D-glucopyranoside (OG), dodecyl- β -D-maltoside (DDM), Fos-choline-12 (FC-12), *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide (LDAO), and 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO). Following extraction from the membrane fraction and a single metal affinity purification step, 21 of the 24 candidates gave an included peak in the gel filtration profiles in at least one detergent (Fig. 2, Table III). Ten of the 24 were found to be monodisperse based on the presence of a single, symmetrical peak (Fig. 2, Supporting Table II). The presence of a monodisperse size exclusion profile has been shown to be a good indicator of membrane protein samples that are of suitable quality for further biochemical, biophysical, and crystallization studies.^{8,9,24–27} It is difficult to

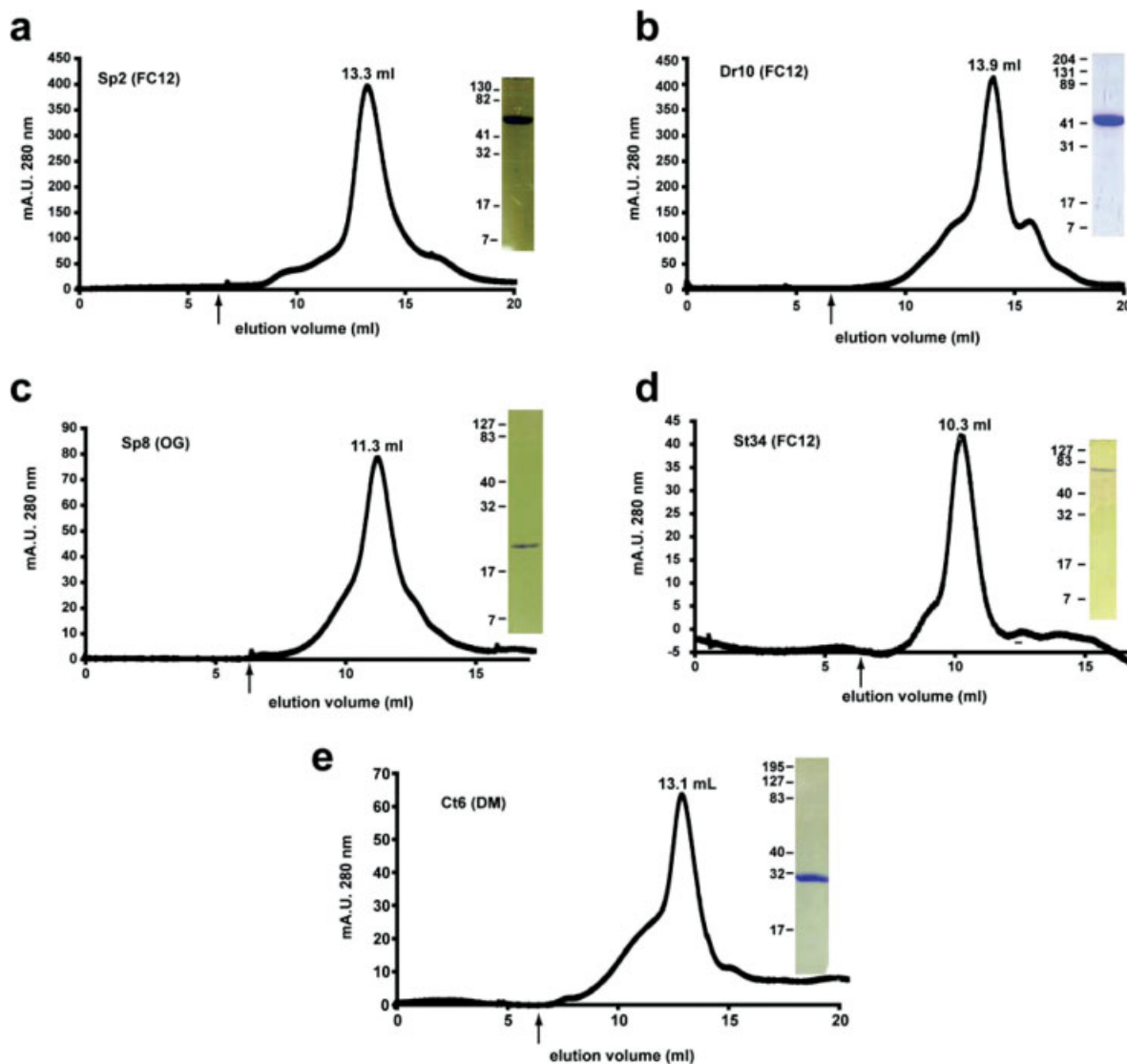


Figure 2. Superdex200 gel filtration profiles and SDS-PAGE analysis of exemplar candidates following batch Ni-NTA purification. The detergent used is indicated in parentheses. Elution volume and coomassie-blue stained SDS-PAGE of the main gel filtration peak fraction are shown. Arrow indicates the void volume. Sample identity, annotation, monomer molecular weight, and approximate size of the observed protein detergent complex size are: (a) Sp2, MscS channel, 49 kDa, ~120 kDa; (b) Dr10, MscS channel, 40 kDa, ~95 kDa; (c) Sp8, MscL channel, 15 kDa, ~180 kDa; (d) St34, ABC transporter, 65 kDa, ~320 kDa; (e) Ct6, proton channel, 27 kDa, ~110 kDa (also see Supporting Table II).

estimate the oligomeric state of a membrane protein from size exclusion results, particularly as the standards are soluble, globular proteins, and the exact biological assembly of the particular proteins we examined is unknown. Nevertheless, we found that all of the monodisperse samples ran with apparent molecular weights that are larger than that expected for monomers. This property was maintained regardless of the detergent type in the cases where more than one detergent yielded a monodisperse peak (Fig. 2, Supporting Table II).

Interestingly, we found that FC-12 was the most successful detergent in terms of both solubilization and behavior in gel filtration. DDM was the second

most effective followed by LDAO, OG, and DM. CHAPSO proved to be least effective at both solubilization and in yielding protein samples having good size exclusion chromatography behavior. The rank order of effectiveness in terms of the ability of specific detergents to produce samples having monodisperse profiles is FC12, DDM, LDAO and is similar to that reported by Drew and colleagues for a recent survey of eukaryotic membrane proteins.⁹

Fos-choline detergents have been used successfully to purify, crystallize, and determine membrane protein structures,^{28,29} for membrane protein NMR studies,³⁰ and for electron microscopy studies of transporter oligomers.³¹ FC12 has further been shown to be

Table III. Detergent Extraction and Gel Filtration Behavior of Overexpressed Extremophile Membrane Proteins

Target	Construct	Induction temp. (°C)	Yield (mg/L)	Detergents						Annotation
				DM	OG	DDM	FC12	CHAPSO	LDAO	
Ct6	HM3C	37	~10	++	–	++	++	–	++	MotA/TolQ/ExbB proton channel
Rm3	H3C	18	6	n.p.	n.p.	n.p.	+	n.p.	n.p.	MotA/TolQ/ExbB proton channel
Gs26	CH	18	1.5	n.a.	n.a.	n.a.	–	n.a.	n.a.	MotA/TolQ/ExbB proton channel
Rm4	CH	18	10	–	n.p.	+	++	–	–	MotA/TolQ/ExbB proton channel
Dr10	H3C	18	9	–	n.p.	++	++	–	–	Mechanosensitive ion channel, MscS
Rm8	CH	18	7	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	Mechanosensitive ion channel, MscS
Sp7	H3C	18	15	–	–	–	++	–	–	Mechanosensitive ion channel, MscS
Rm11	H3C	18	7	–	n.p.	–	+	–	+	Mechanosensitive ion channel, MscS
Sp2	H3C	18	11	–	n.p.	–	++	–	–	Mechanosensitive ion channel, MscS
Sp8	H3C	25	2	–	++	++	++	–	++	Mechanosensitive ion channel, MscL
Dr35	CH	18	0.5	n.p.	n.a.	n.a.	+	n.a.	n.a.	Multidrug efflux transporter
Rm29	H3C	18	7	n.p.	n.p.	n.p.	++	n.p.	–	High-affinity Nickel transporter
Sp1	H3C	25	20	–	–	+	+	–	–	Voltage-gated sodium channel subunit
So15	H3C	25	>10	–	n.p.	+	+	n.p.	n.p.	Iron compound, ABC transporter, permease
So22	H3C	18	<1	n.a.	n.a.	n.a.	+	n.a.	n.a.	Sulfate ABC transporter, permease
Gs6	HM3C	25	~5	+	+	+	–	n.p.	+	Heavy metal efflux pump
St34	CH	18	1	–	n.a.	n.a.	++	n.a.	n.a.	Lipid/multidrug/protein-type ABC exporter
Tm23	H3C	18	~0.5	n.p.	n.a.	n.a.	+	n.a.	n.a.	Na ⁺ /H ⁺ antiporter
Af11	HM3C	25	6	n.p.	n.p.	+	+	+	n.p.	Potassium channel
Bh8	H3C	37	12	–	–	–	–	–	–	Potassium channel
Af27	CH	18	10	–	+	–	–	–	–	No significant homologues
Ph11	CH	18	10	+	+	+	+	–	++	Na ⁺ /solute symporter
So25	HM3C	18	8	+	n.p.	+	+	–	+	Multidrug resistance
Dr18	CH	18	1	n.a.	++	++	++	n.a.	n.a.	Multidrug resistance

List of purified candidates showing several parameters including type of construct, induction temperature, yield/L, and gel-filtration behavior in selected detergents. ++, monodisperse peak; +, good gel-filtration profile; –, poor gel-filtration profile; n.p., no gel-filtration profile; n.a., not tested.

efficient at membrane protein solubilization and at yielding monodisperse samples as judged by size exclusion chromatography.⁷ Nevertheless, concerns have been raised that even though Fos-choline detergents are efficient at extracting proteins from membrane, they may be harsh enough to need to be treated with caution²⁷ and may not always yield a stable sample.³² It is notable that 11 of the 19 proteins that gave good gel filtration profiles in FC12 also gave similar gel filtration profiles in at least one other more mild detergent. This result suggests that, at least in these cases, the samples were not likely to be simply solubilized and denatured by FC12.

Our detergent extraction and gel filtration experiments identify many candidates that were able to produce protein that gave good gel filtration behavior in at least one detergent following a very simple purification protocol (21/24 candidates) (see Fig. 4). This observation provides further support for the idea that the original GFP screen was a good proxy for identifying membrane proteins that have the potential to yield material of suitable quality for biophysical experiments.

Discussion

Although there are now a number of reports in which GFP-based screens have been used to identify proteins or constructs that have appropriate biophysical characteristics for structural biology experiments,^{3,5–9} there

have remained some questions regarding the fidelity of the assay. Our results establish that there is an excellent correspondence between the behavior of membrane protein GFP-fusions and the potential of a given candidate to be overproduced. Of the candidates that pass the screen and were subjected to further characterization, 90% can be produced at ≥ 0.5 mg L⁻¹ of culture, with half of these made at ≥ 5 mg L⁻¹. These expression scales are suitable for investing further effort into purification, biophysical characterization, and crystallization trials. An important feature of this work is that the screening can be done manually and requires no specialized robotic equipment. Thus, such screens should be applicable in a wide range of settings.

Membrane protein expression: influence of molecular weight, transmembrane segment number, and codon usage

Our survey of the expression behavior of a large number of membrane proteins from diverse sources provides a dataset that yields insight into the factors that influence the ability to overproduce a candidate membrane protein [Fig. 3(a,b)]. There are some clear general lessons regarding the likelihood of success for a candidate based on a few simple properties. In general, the total molecular weight of the candidate appears to be an important factor. We found many well expressed membrane proteins in the 20–50 kDa

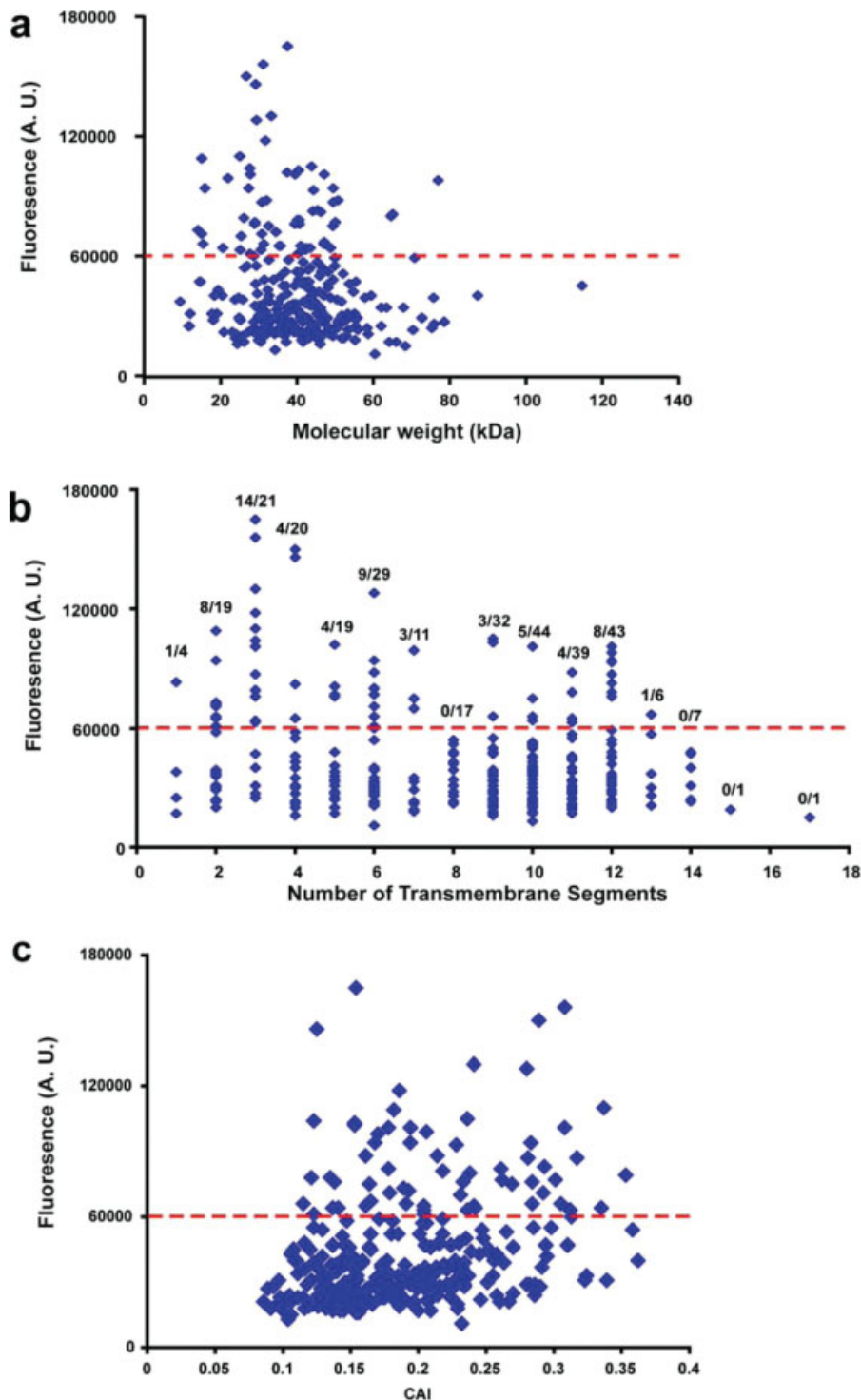


Figure 3. Distribution of MW, TMs, and codon usage among the GFP-screened candidates with respect to their fluorescence scores. (a) Molecular weight, (b) Predicted number of transmembrane helices. Fraction expressed per class: 1 TM (25%), 2 TM (42%), 3 TM (67%), 4 TM (20%), 5 TM (21%), 6 TM (31%), 7 TM (27%), 8 TM (0%), 9 TM (9%), 10 TM (11%), 11 TM (10%), 12 TM (18%), 13 TM (17%), 14 TM (0%), 15 TM (0%), 17 TM (0%). (c) CAI for each gene, calculated using RSCU values from the class II highly-expressed *E. coli* gene reference set.³³ Higher CAI values correspond to better matches with the host codon usage.

range, (59/262, 22.5%), but very few in the >50 kDa range (5/52, 9.6%). Similarly, there is a clear difference in the success rates with respect to the number of

predicted transmembrane segments; ~35% of the 123 candidate proteins having 1–7 transmembrane express better than the cutoff whereas only 11% of the 191

proteins having 8–17 transmembrane segments expressed well. It is particularly striking that 67% of the proteins having three predicted transmembrane segments expressed as well as or better than the benchmark proteins.

There is a general notion that one of the parameters that may have an impact on the ability to overexpress a candidate protein in *E. coli* is how suited the codon usage of the candidate gene is to well-expressed proteins from host organism.^{34,35} However, data supporting this belief are equivocal. There are anecdotal reports of successes,^{36,37} failures,³⁸ and variable outcomes³⁹ for particular cases of codon optimization. Large scale study of a set of 1000 soluble proteins from *Plasmodium falciparum*, an organism with proteins that are known to be particularly challenging to express in *E. coli*, found no significant correlation with codon usage.⁴⁰ As we had a diverse set of proteins from many organisms that have a range codon usages, we asked whether there was any correlation between how well the candidate proteins expressed and the similarity of their codon usage to that of well-expressed *E. coli* genes as measured by the “codon adaptation index,” CAI. This parameter gives a length independent assessment of how well each test gene matches the codon usage of well-expressed proteins from the host.³³ Strikingly, for those candidates that pass our threshold GFP value, we find no correlation between the CAI for a given gene and expression level [Fig. 3(c)]. Indeed, a number of the proteins having the highest GFP values can be produced at very high levels following optimization in spite of having very low CAI scores (Ph11, CAI = 0.123, 10 mg L⁻¹; So15, CAI = 0.153, >10 mg L⁻¹; Bh8 CAI = 0.154, 12 mg L⁻¹). Even though individual rare codons may certainly cause expression difficulties,^{34,35} our data support the conclusion that codon optimization is not a key parameter for heterologous membrane protein expression in *E. coli*.

Comparisons other GFP-membrane protein screens

Given the challenges associated with membrane protein biochemistry, there have been a number of reports describing the implementation of GFP-based screens as a means to identify potentially tractable candidate proteins. Although large scale GFP screens in *E. coli*,⁶ *S. cerevisiae*⁹ have reported success in identification of well-expressed candidates, multi-milligram expression levels of non-host derived proteins per liter of culture have not yet been generally demonstrated. A key advance reported here is the use of benchmark proteins to set a threshold level for well-expressed candidates paired with construct and expression temperature optimization protocol. Together, this approach identifies a large number of candidate proteins that can be made at ≥ 0.5 mg L⁻¹ with some candidates expressing better than 10 mg L⁻¹.

Similar to the recently reported *S. cerevisiae* survey,⁹ we find that Fos-choline 12 (FC-12) has the best success at both solubilizing membrane expressed material and producing material having a good gel filtration profile (Table III). The high success with fos-choline detergents in membrane protein solubilization has raised some of the concern regarding the potential destabilizing effects of this detergent class.^{27,32} Importantly, a large number of the expressed extremophile membrane proteins that can be extracted from the membrane using FC-12 are also extracted from the membrane and give good gel filtration profiles in other, more conventionally used detergents.

Methods

Target selection and sequence analysis

Annotation searches for membrane proteins denoted as ion channels, transporters, exchangers, antiporters, carriers and major facilitator superfamily in the genomes of *Aeropyrum pernix* (Ap), *Archaeoglobus fulgidus* (Af), *Bacillus halodurans* (Bh), *Chlorobium tepidium* (Ct), *Deinococcus radiodurans* (Dr), *Desulfovibrio vulgaris* (Dv), *Geobacter sulfurreducens* (Gs), *Oenococcus oeni* (Oo), *Pyrococcus furiosus* (Pf), *Pyrococcus horikoshii* (Ph), *Ralstonia metallidurans* (Rm), *Shewanella oeneidensis* (So), *Silicibacter pomeroyi* (Sp), *Streptococcus thermophilus* (St), *Sulfolobus solfataricus* (Ss), *Thermotoga maritima* (Tm), *Thermus thermophilus* (Tt), and *Thermoplasma volcanium* (Tv) were performed using the Integr8 protein database (<http://www.ebi.ac.uk/integr8>). To find unannotated the membrane proteins, each genome was searched with a sequence representing a generic transmembrane helix using BLASTP⁴¹ at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>). This search yielded a list of membrane proteins in the chosen organism from which we selected several proteins with no homologues of known structure. All transmembrane helix predictions were performed with TMHMM 2.0¹⁶ (<http://www.cbs.dtu.dk/services/TMHMM-2.0>). A complete list of screened proteins and values from the GFP screen can be found in Supporting Table I.

Construct generation

All genes were cloned using ligation-independent cloning (LIC)¹³ of PCR products containing LIC overhangs and directly cloned into vector containing a complementary LIC cassette (Supporting Fig. 1). For the GFP screening, genes encoding candidate membrane proteins were cloned into a pET-based LIC vector that harbors a Tobacco etch virus protease cleavage site followed by a C-terminally His₈ tagged GFP. The three vectors used in overexpression optimization experiments were H3C (N-terminal His₈ tag-3C protease cleavage site⁴² followed by the gene of interest), HM3C (N-terminal His₆ tag-maltose binding protein-3C protease cleavage site followed by the gene of

interest), and CH (gene of interest followed by a TEV protease cleavage site and a His₈ tag). The GFP and CH vectors share the same LIC cassette, whereas, the H3C and HM3C vectors share a different LIC cassette (Supporting Fig. 1). PCR reactions were done using Phusion high-fidelity DNA polymerase (Finnzymes) and genomic DNA for each parent organism. Genomic DNA was obtained from ATCC (www.atcc.org).

Expression of GFP-fusion and non-GFP fusion membrane proteins

Two different cell types of *E. coli* were used. BL21 (DE3) pLysS were used for screening of GFP-fusion candidates and C41 cells⁴³ were used for expression optimization and protein production. BL21 (DE3) pLysS cells were used for the GFP screen owing to the observation that they are reported to be less sensitive to variations in OD_{600 nm} of induction than the C41 strain.⁷

GFP screening

Individual *E. coli* BL21 (DE3) pLysS cultures for each of the candidate GFP-fusions were grown in 7 mL Luria-Bertani (LB) media at 37°C to OD_{600 nm} ~0.5. Induction was done at 25°C using 0.4 mM IPTG. After 20 h of growth post-induction, the cell pellet was recovered from 1 mL of culture (2 min at 13,000 rpm in a bench top 5415D centrifuge (Eppendorf)) and resuspended in 200 µL of 200 mM KCl, 20 mM Tris pH 8.0. Samples were subjected to the whole-cell fluorescence assay (465–485 nm excitation, 515–575 nm emission) using a 20/20ⁿ luminometer with a Blue fluorescent module (Turner Biosystems, U.S.A.). In each experiment uninduced cells and cells expressing the GFP fusion of BH8, a well-expressed candidate protein, were run as controls. These controls have reproducible fluorescence measurements over a number of trials, F.A.U. of 23,667 ± 1021, *n* = 6, and 174,964 ± 5397, *n* = 6, for uninduced and BH8 expressing cells, respectively.

Expression optimization using non-GFP constructs

Proteins for overexpression optimization were selected based on the fluorescence values from the GFP screen. Genes for the selected proteins were cloned into H3C, CH, and HM3C tag formats and transformed into C41 cells.⁴³ Cultures were grown in 7 mL LB media at 37°C. Induction was performed at OD_{600 nm} ~0.5 using 0.4 mM IPTG. Immediately following induction, cultures were moved to one of three different expression temperatures, 18°, 25°, or 37°C, and were grown for 48, 20, and 3 h, respectively. Following expression, 500 µL of cells were harvested by centrifugation (2 min at 13,000 rpm in a bench top 5415D centrifuge (Eppendorf)), resuspended in 50 µL of 200 mM KCl, 20 mM Tris, pH 8.0, and lysed by the addition of 50 µL of 2% SDS, 20% glycerol, 0.05% bromophenol

blue, 125 mM Tris pH 6.8. Expression was assayed by SDS-PAGE analysis with Coomassie staining and Western blot using an anti-His₅ HRP conjugate antibody (Qiagen). The best-optimized condition from this assay was used for a scale-up.

Membrane preparations

C41 *E. coli* cells harboring the expression construct of interest were grown in 1 L 2xYT (5 g NaCl, 16 g tryptone, 10 g yeast extract) under conditions that were determined to give the best expression for each individual construct under investigation. Cell pellets obtained from the 1 L culture were suspended in 90 mL lysis buffer (200 mM KCl, 20 mM Tris pH 8.0, 8% glycerol, 2 mM EDTA, 1 mM PMSF, 1 mM DTT) and disrupted by sonication for 4 min on ice using a Sonic Dismembrator model 500 (Fisher). Cell lysate was centrifuged in a JA 25.50 rotor (Beckman Coulter) at 7000g for 35 min at 4°C to remove unbroken cells and debris. The supernatant was then centrifuged in a Ti45 rotor (Beckman Coulter) at 40,000 rpm for 2 h at 4°C to separate the membrane pellet. Pellets were homogenized in a storage buffer (200 mM KCl, 20 mM Tris pH 8.0, 8% glycerol) with a Dounce Tissue Grinder (Kimble Kontes LLC) and frozen at -80°C.

Membrane protein extraction, purification, and characterization

All extraction, purification, and characterization were done at 4°C. Membrane extractions and purifications were performed with six detergents (Anatrace, USA): DM (40 mM), OG (200 mM), LDAO (2% v/v), DDM (20 mM), CHAPSO (160 mM), and FC12 (40 mM), added and incubated for 2 h at 4°C. Detergent-solubilized membrane proteins were separated from insoluble material by ultracentrifugation for 45 min at 55,000 rpm at 4°C in a TLA 110 rotor on a bench-top ultracentrifuge (Beckman Coulter). Following recovery, the supernatant was incubated with a bed volume of 0.5 mL of pre-equilibrated Ni-NTA superflow beads (Qiagen) at 4°C. Protein-bound beads were washed with the appropriate detergent buffer consisting of 200 mM KCl, 30 mM imidazole, 8% glycerol (v/v), detergent (3× critical micelle concentration (CMC), except for LDAO which was used at 10× CMC), 20 mM Tris, pH 8.0 and eluted with the same detergent buffer containing 300 mM imidazole. The eluted protein samples were >80–90% pure based on SDS-PAGE analysis. Protein concentration was determined by absorbance at 280 nm.⁴⁴ Purified samples were subjected to size exclusion chromatography on a Superdex200 column (GE Healthcare) to assess the homogeneity and stability in a running buffer of 200 mM KCl, 8% glycerol (v/v), detergent (3× CMC, except for LDAO which was used at 10× CMC), 20 mM Tris, pH 8.0.

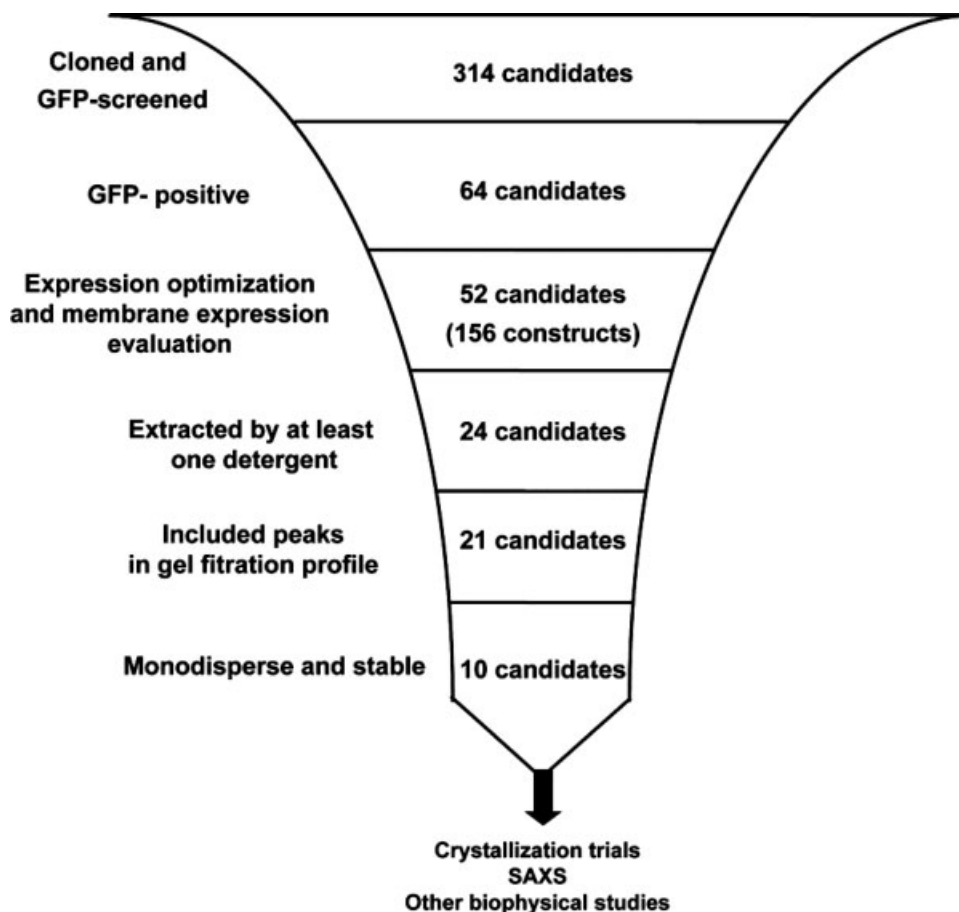


Figure 4. Summary of extremophile membrane protein GFP screen. Results are presented as a funnel. At each stage some candidates are eliminated due to a failure to pass progressively more stringent tests. The exception is the step from GFP-positive to expression optimization in which 32 GFP-positive proteins along with 20 representatives from categories having lower GFP signals were chosen for expression optimization and further testing. Candidates passing the final stage of being monodisperse and stable are suitable for immediate evaluation by crystallization trials, small-angle X-ray scattering (SAXS), or other biophysical and functional analysis.

Codon adaptation index calculation

Codon adaptation index (CAI) values for each candidate gene were calculated according to Sharp and Li³³ using the webserver (<http://www.evolvecode.net/codon/cai/cais.php>).⁴⁵ $CAI = CAI_{obs}/CAI_{max}$ where the observed CAI for a given gene sequence $CAI_{obs} = (\prod_{k=1}^L RSCU_k)^{1/L}$ and the maximal CAI score is $CAI_{max} = (\prod_{k=1}^L RSCU_{kmax})^{1/L}$. $RSCU_k$ is the “relative synonymous codon usage” value for the k th codon in the gene, $RSCU_{kmax}$ is the maximum RSCU value for the amino acid encoded by the k th codon in the gene, and L is the number of codons in the gene.

$RSCU_{ij} = x_{ij} / \frac{1}{n_i} \sum_{j=1}^{n_i} x_{ij}$ where x_{ij} is the number of occurrences of the j th codon for the i th amino acid and n_i is the number (from 1–6) of alternative codons for the i th amino acid. $RSCU_{kmax}$ values were calculated from the class II highly-expressed *E. coli* gene reference set.³³

Conclusions

Producing membrane proteins and membrane protein complexes for biochemical and structural study remains a major challenge. The development of methods that provide a rapid way to identify membrane proteins that can be expressed in sufficient amounts and of sufficient quality is an area of intensive effort.^{4,6–9,46} This report provides strong evidence that screening GFP-fusions provides a fruitful approach for identifying well-expressed candidate membrane proteins that display favorable biophysical behaviors suitable for further functional and structural investigation, and that a key factor in success is the investment in optimization of construct tags and induction temperature.

Structural and biochemical studies of integral membrane proteins remains challenging because identifying a candidate protein that has good expression, that can be readily purified, and that has suitable behavior in detergent solution can require a

substantial investment in time and resources. By combining a GFP-screen with a simple optimization protocol, we have been able to achieve a high success rate identifying candidate proteins that can be well expressed and that display desirable biophysical characteristics. A key implementation was the use of benchmark proteins to set a level of GFP fluorescence that will identify a candidate with a high likelihood of success. We anticipate that the large number of diverse membrane proteins we identify here as having good expression profiles will provide excellent starting points for both functional and structural studies. A summary of the outcome of the screen is shown in Figure 4. As with other expression surveys of panels of membrane proteins in which multiple levels of behavior are evaluated,²⁷ the approach we report here functions as a funnel in which there is an attrition of candidates as the stringency imposed at each step increases. It may be possible to salvage some failed candidates by additional exploration of alternate constructs that are designed to eliminate regions of predicted polypeptide disorder. Further, just as combination of mutagenesis and GFP screens has proved useful for defining appropriate constructs of soluble proteins for crystallization and structure determination,^{47,48} it is likely similar combination mutagenesis/GFP screens should prove fruitful for enhancing the production of crystal grade membrane protein samples or for rescuing failed candidates. Similarly, GFP-based studies using other expression hosts and as a means to identify well-expressed complexes through co-expression strategies^{49–51} should enable the structural investigation of an array of membrane proteins and membrane protein complexes.

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