An efficient strategy for high-throughput expression screening of recombinant integral membrane proteins

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Abstract

The recombinant expression of integral membrane proteins is considered a major challenge, and together with the crystallization step, the major hurdle toward routine structure determination of membrane proteins. Basic methodologies for high-throughput (HTP) expression optimization of soluble proteins have recently emerged, providing statistically significant success rates for producing such proteins. Experimental procedures for handling integral membrane proteins are generally more challenging, and there have been no previous comprehensive reports of HTP technology for membrane protein production.

Here, we present a generic and integrated parallel HTP strategy for cloning and expression screening of membrane proteins in their detergent solubilized form. Based on this strategy, we provide overall success rates for membrane protein production in *Escherichia coli*, as well as initial benchmarking statistics of parameters such as expression vectors, strains, and solubilizing detergents. The technologies were applied to 49 *E. coli* integral membrane proteins with human homologs and revealed that 71% of these proteins could be produced at levels that allow milligram amounts of protein to be relatively easily purified, which is a significantly higher success rate than anticipated. We attribute the high success rate to the quality and robustness of the methodology used, and to introducing multiple parameters such as different vectors, strains, and detergents. The presented strategy demonstrates the usefulness of HTP technologies for membrane protein production, and the feasibility of large-scale programs for elucidation of structure and function of bacterial integral membrane proteins.

Keywords: detergent screen; high-throughput; membrane proteins; multiparameter; parallel screen

Membrane proteins play major roles in many biological processes such as signaling, metabolism, solute and macromolecular transport, and bioenergetics. Therefore, they are also major pharmaceutical targets. However, a deeper understanding of structure–function relationships of membrane proteins requires high-resolution structural information. To date, the Protein Data Bank (PDB) contains >26,000 structures, of which ~50 are annotated as distinct integral membrane proteins (http://www.rcsb.org/pdb/). Considering that 20%–25% of all proteins in a typical cell are integral membrane proteins, the number of known membrane protein structures obviously represents only a very small fraction of all existing proteins.

To allow various structural studies, tens or even hundreds of milligrams of highly purified protein might be needed. Thus, an efficient recombinant overexpression system for membrane proteins is in most cases a prerequisite. However, membrane proteins are not only difficult to express, but also difficult to isolate, since they require purification in detergent. Furthermore, the very hydrophobic nature of integral membrane proteins makes them hard to handle ex-

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Abbreviations: BL21, BL21(DE3); C41, C41(DE3); C43, C43(DE3); DDM, dodecyl maltoside; *E. coli, Escherichia coli*; FC12, FOS-CHOLINE-12; HTP, high-throughput.

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perimentally, and they can easily be "lost" upon purification, centrifugation, or gel electrophoresis. General experiences from workers in the field with the problematic experimental behavior of integral membrane proteins have lead to the expectation that these proteins are dramatically harder to produce than soluble proteins.

Presently, there are intensive ongoing efforts in development of high-throughput (HTP) expression technology for soluble protein production with success rates in the range of 50%–60% of bacterial target genes entered into the process (Kuhn et al. 2002; Lesley et al. 2002; Stewart et al. 2002; Hui and Edwards 2003). So far no HTP technologies or comprehensive success rates for membrane protein production have been reported. At present, the most extensive efforts for bacterial membrane protein production involve transporter proteins (for review, see Loll 2003). However, in order to produce sufficient amounts of such proteins for structural studies, large-scale fermentors of up to 50 L have been used (Henderson et al. 2000; Chang and Roth 2001).

An additional complication in membrane protein production is the detergent extraction procedure. The choice of detergent is a critical issue to consider, especially when designing HTP studies. There are dozens of different detergents that are commonly used, dozens more that are less characterized but still probably useful, and many novel detergents under development. Moreover, mixtures of detergents are sometimes used (Koronakis et al. 2000). It has also been reported that some compartments of the cell membrane show resistance toward certain detergents (Schuck et al. 2003). Altogether, the size of the detergent parameter space becomes very large.

Therefore, to allow larger numbers of integral membrane proteins to undergo extensive expression and purification screening, efficient parallel technology is urgently needed (see also Lundstrom 2004). To this end, we present a versatile multiparameter HTP strategy for cloning and expression screening of membrane proteins from *Escherichia coli* homologous to those from *Homo sapiens*. Furthermore, an efficient HTP detergent screen is presented. Based on these technologies, we derive initial benchmarking statistics for the production of bacterial membrane proteins in *E. coli*, using multiple expression vectors, strains, and conditions. We believe that the type of strategy presented is a first step toward more routine structure determination of membrane proteins, and thereby toward the generation of a wealth of information on the processes of the biomembranes, many with critical biomedical importance.

Results

Protein target selection and gene cloning using Gateway technology

Overexpression of membrane proteins in mammalian expression systems has so far not been very useful for the production of proteins of enough quality for structural studies (Tate 2001). However, a bacterial protein can be used as a prototype to obtain the essential information about the structure and mechanism of its mammalian homolog. Therefore, to create a target list of *E. coli* membrane proteins, a search for proteins that have both human and *E. coli* analogs, and that are found in Protein Families (PFAM) but not in PDB databases, was performed. The search produced 703 hits, including members of 48 membrane protein families. Further narrowing the search yielded 49 members from 39 families (such as transporters, ion channels, permeases, and transferases), most of which had at least four predicted transmembrane domains.

The two-step recombination cloning of 49 gene targets into three expression vectors using Gateway technology yielded 47 types of constructs (two cloning reactions failed), each with either a 6-His-, a FLAG-, or an MBP- at the NH_2 terminus and a 6-His tag coding sequence followed by three stop codons at the COOH terminus (Table 1). The presence of a C-terminal 6-His tag in all constructs allowed dot-blot detection of all proteins using anti-6-His probe. The constructs were successfully used to transform C41, C43, and BL21 strains.

HTP detection of expressed membrane proteins in 96-well plates

Using a 96-deep-well plate, the positive clones were grown simultaneously. Low temperature and low IPTG concentration had a positive effect on the production of membrane-targeted proteins in our experiments (data not shown); an observation also made by others (Wang et al. 2003). A common method for isolation of membrane proteins is ultracentrifugation of vesicles following cell lysis, which is by no means a HTP method. Hence, we decided to investigate the feasibility of a HTP screen using direct detergent extraction of membrane proteins in the noncentrifuged cell lysate, followed by affinity purification. The solubilized material was successfully cleared from cell debris by filtration in the 96-well format. To distinguish between solubi-

Table 1. *Summary of results for HTP membrane protein expression*

	Number	Success rate
Targets	49	100%
Positive clones	47	96%
Positive expression[a]	35	71%
Expression: C41-HIS	26	53%
Expression: C41-MBP	23	47%
Expression: C41-FLAG	24	49%
Expression: C43-FLAG	22	45%
Expression: BL21-FLAG	25	51%

[a] Describes the total number of purified proteins having been expressed in at least one construct.

lized membrane proteins and membrane fragments or vesicles in the filtrate, a second step involving His-tag purification in 96-well filter plates was introduced. To allow initial detection of all purified proteins, we decided to perform dot-blot analysis rather than SDS-PAGE, since the latter is less efficient as a HTP method. Dot-blot analysis of the eluted material showed the expression of 26 proteins with N-terminal 6-His-tag, 24 proteins with N-terminal FLAG-tag (Fig. 1) and 23 proteins with N-terminal MBP-tag in C41 cells. Thus, in these cells a total of 31 distinct proteins were expressed in at least one construct (Table 2). To investigate the importance of the expression strain, the FLAG-tagged expression constructs were transformed into C41, C43, and BL21 cells. The C41 and C43 strains have been derived from BL21 to increase the yield of the membrane-targeted proteins (Miroux and Walker 1996). In total, 29 FLAG-tagged proteins were expressed: 22 in C43, 24 in C41, and 25 in BL21 (Table 2). Hence, the number of all the proteins expressed in at least one vector and strain was increased to 35 after combining all parameters (Tables 1, 2). Based on medium-scale production experiments (see below) we estimated expression levels to be between 0.2 and 5 mg protein per liter shake flask culture.

Detergent screening

Using the HTP screen, we investigated the effect of a number of various detergents in extraction and purification of 12 selected proteins. A selection of 25 detergents from nine different families was made (Table 3). These detergents either belong to families whose members have been successfully used to produce crystals, such as maltosides and glucosides (Reiss-Husson and Picot 1999), or are commonly used for protein purification in many laboratories, such as Triton and CHAPS. The amounts of detergents used during extraction and purification procedures were chosen based on the CMC for each detergent, but the detergent concentration was never <1% during the extraction. Certain detergents, such as CHAPS, may require >2% for efficient solubilization. However, we did not exceed the final detergent concentration of 2%, since that is the maximum concentration recommended for Ni-NTA agarose purification. In order to have equal starting conditions, culture growth and induction of expression were performed in shake flasks prior to transfer into 96-deep-well plates for harvest. Using the 96-well format purification described above followed by dot-blot screen, we could compare the efficiency of different detergents and identify the most suitable one for the extraction and purification of each protein (Fig. 2). Interestingly, the extraction and purification efficiency was quite the same for members of the same detergent family. Some proteins, like EM23 and EM35, could be efficiently extracted by nearly all detergents. In the case of EM43, however, FC10–FC12 were the only detergents able to solubilize high amounts of the protein (Fig. 2F). In fact, extraction and purification with FC12 always resulted in high amounts of purified proteins. Therefore, we decided to use FC12 as the standard detergent to screen for expression of all the proteins (see Fig. 1). The detergents in this series have the same head groups as phospholipids, but unlike the phospholipids they possess simple hydrophobic tails. Maltosides and thiomaltosides also resulted in high purification yield. The maltose head group is believed to increase the solubility of the members within the family as compared to the glucoside family. The maltosides are, therefore, still mild, but more efficient detergents than the glucosides.

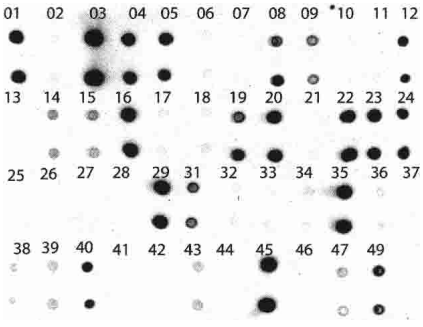

Figure 1. Dot-blot of purified FLAG-tag proteins overexpressed in C41 cells. Two different colonies were used to produce each protein. All the proteins were extracted with FC12.

Evaluation of expression results by medium-scale protein production

Seven proteins—three expressing at high, two at medium, and two at low levels—were selected for medium-scale purification, in order to determine the yield and to verify the expression and the homogeneity of the target protein. Figure 3 shows the intactness and the homogeneity of the proteins purified on gel filtration with either FC12 or DDM. The amounts of purified proteins after gel filtration were calculated to be 3–5 mg/L culture for high expression, 1–3 mg/L for medium expression, and ~0.2–1 mg/L for low expression proteins. Interestingly, the integrity of the purified proteins seems to depend on the nature of the detergent used. Some proteins tend to aggregate in the presence of FC12 (a zwittergent) but not in the presence of DDM (a neutral detergent), and vice versa (Fig. 4). Most proteins were shown to be homogenous in at least one detergent, while EM43 was the only protein that showed to be rather heterogeneous, although it is possible to separate different populations by gel filtration (Fig. 3F). EM43 is predicted to be a Co^{2+}/Mg^{2+} channel based on amino acid sequence analysis. When $MgCl_2$ was added to all the buffers during the purification EM43 was eluted as a major sharp band, corresponding to either its largest oligomeric form or nonhomog-

Table 2. *Protein targets used in the study and expression levels of the corresponding constructs*

EM No.	Swiss P. No.	Predicted function	Cloned	C41-HIS	C41-MBP	C41-FLAG	C43-FLAG	BL21-FLAG
01	P27837	Probable transporter	Yes	+++	++	+++	+++	++
02	P77265	Multidrug resistant	Yes	–	–	–	–	–
03	P37905	Ammonium transport	Yes	+++	+++	+++	+++	+++
04	P75757	Zinc transport	Yes	++	+++	++	+++	+++
05	P06978	PGP synthase	Yes	++	+	++	+++	+
06	P76091	Unknown	Yes	–	–	–	–	–
07	P45394	Unknown	Yes	–	–	–	–	–
08	P76007	Cell volume regulation	Yes	+++	+++	++	++	+++
09	P32683	Unknown	Yes	+	–	+	+	–
10	P77405	Citrate transport	Yes	–	–	–	–	–
11	P77145	Mn^{2+} transport	Yes	–	–	–	–	–
12	P39276	Probable transporter	Yes	+++	++	++	+	+++
13	P42602	Probable transporter	Yes	–	++	–	–	–
14	P09836	Regulatory protein	Yes	+++	+	+	+	+
15	P16256	Pantothenate permease	Yes	+++	+	+	+	+
16	P31462	Probable transporter	Yes	+++	+++	+++	+++	+++
17	AAA99171	prs-associated protein	Yes	–	–	–	–	–
18	P26601	Polyprenyltransferase	Yes	+	–	–	–	–
19	P06967	Unknown	Yes	+++	+	++	+	–
20	P76526	Ion transport	Yes	+++	+++	+++	+++	++
21	P24198	Ion transport	Yes	–	–	–	–	+++
22	P33780	Transporter	Yes	+++	++	+++	+++	++
23	P33021	Probable transporter	Yes	–	–	+++	+	+++
24	P31125	Efflux pump	Yes	–	–	++	–	–
25	P37308	Phosphate transport I	Yes	–	–	–	–	–
26	P23894	Probable protease	Yes	–	–	–	–	–
27	P33031	Nucleoside permease	Yes	–	+	–	–	–
28	P28303	DNA-damage inducible	Yes	–	–	–	–	+
29	P09391	Unknown	Yes	+++	+++	+++	+++	++
30	P23889	Polysialic acid transport	No	–	–	–	–	–
31	P03844	Preprotein translocase	Yes	++	++	++	+	++
32	P22520	ATP-binding protein	Yes	–	+++	–	–	–
33	P43676	Phosphate transport II	Yes	–	–	–	–	+
34	P15876	Phosphotransferase	Yes	+	–	–	–	–
35	P25714	Unknown	Yes	+++	+++	+++	+++	+++
36	P76473	Unknown	Yes	–	–	–	+	+++
37	P13974	Unknown	Yes	–	–	–	–	–
38	Q46827	Unknown	Yes	+	–	–	–	+
39	P27304	Multidrug resistance	Yes	++	–	+	–	–
40	P32705	Probable transporter	Yes	+++	+++	++	+++	+++
41	P16920	Preprotein translocase	Yes	–	–	–	–	–
42	P27854	Probable biosynthesis	Yes	++	+	–	–	–
43	P27841	Mg^{2+}/Co^{2+} transport	Yes	++	–	+	–	+
44	P31071	Cardiolipin synthase	Yes	–	–	–	–	–
45	P77304	Peptide permease	Yes	+++	+++	+++	+++	+++
46	P21345	Glutamate transport	Yes	–	–	–	–	–
47	P77610	Asparagine permease	Yes	+	+	+	+	++
48	P27844	Unknown	No	–	–	–	–	–
49	Q8X7L9	Tyrosine kinase	Yes	++	++	++	++	+

High expression (+++), medium expression (++), low expression (+), no expression (–).

enous aggregates (data not shown). Gel filtration of EM47 resolved two major populations from each other (Fig. 3G). EM47 was not present in the void fraction and came as a separate peak, probably a tetramer. The void, however, contains an unknown protein at a higher oligomeric/aggregation form.

Discussion

Using the presented generic strategy for cloning and expression screening of bacterial integral membrane proteins, we could express 71% of the proteins in our target list, and the scale-up experiments indicate that a large fraction of these

Table 3. *List of the detergents and their concentrations used in the screen*

Detergent name	Abbrev.	CMC (mM)	Extraction (mM)
FOS-CHOLINE-9	FC9	39.5	66 (2%)
FOS-CHOLINE-10	FC10	11	63 (2%)
FOS-CHOLINE-11	FC11	1.85	30 (1%)
FOS-CHOLINE-12	FC12	1.5	32 (1%)
HEGA-10	HEGA-10	7	54 (2%)
Nonyl Maltoside	NM	6	43 (2%)
Decyl Maltoside	DM	1.8	21 (1%)
Undecyl Maltoside	UDM	0.59	20 (1%)
Dodecyl Maltoside	DDM	0.17	20 (1%)
CHAPS	CHAPS	8	32 (2%)
CHAPSO	CHAPSO	8	32 (2%)
Nonyl ThioMaltoside	NTM	3.2	32 (1.5%)
Decyl ThioMaltoside	DTM	0.9	20 (1%)
Undecyl ThioMaltoside	UDTM	0.21	19 (1%)
Dodecyl ThioMaltoside	DDTM	0.05	1.9 (1%)
LDAO	LDAO	1	43 (1%)
TDAO	TDAO	0.29	39 (1%)
C8E4	C_8E_4	8	64 (2%)
C8E6	C_8E_6	10	51 (2%)
C10E5	$C_{10}E_5$	0.81	26 (1%)
C12E8	$C_{11}E_8$	0.09	19 (1%)
Octyl Glucoside	OG	18	68 (2%)
Nonyl Glucoside	NG	6.5	65 (2%)
Triton X-100	TX100	0.23	15 (1%)
Triton X-114	TX114	0.20	36 (2%)

should be possible to purify in a folded and homogenous form. Therefore, this success rate for producing bacterial integral membrane proteins approximates those that have so far been obtained for producing bacterial soluble proteins. Although, there were no correlations between the expression and function of the target proteins, we noticed that the majority of the expressed proteins had a high number of transmembrane helices: 60% of the expressed proteins had eight transmembrane helices or more. The cause of the high success rate is probably a combination of different factors. The technology implemented is apparently very robust. The expression vectors have a low noninduced background expression due to an extra repressor site (Tobbell et al. 2002). The *att*-sites introduced by the Gateway cloning clearly do not result in any problems. Also, the use of tags both in the N- and C-termini might have some protective properties. This platform includes a number of reagents and materials that have been carefully evaluated in the process of establishing the technology. Another important factor is the versatile multiparameter approach used. In this study, we performed parallel screens of three vectors and three strains; studies of the effects of temperature, induction time, and inducer concentrations have been performed on selected sets of proteins (data not shown). Moreover, by screening 25 different detergents, we were able to select the most

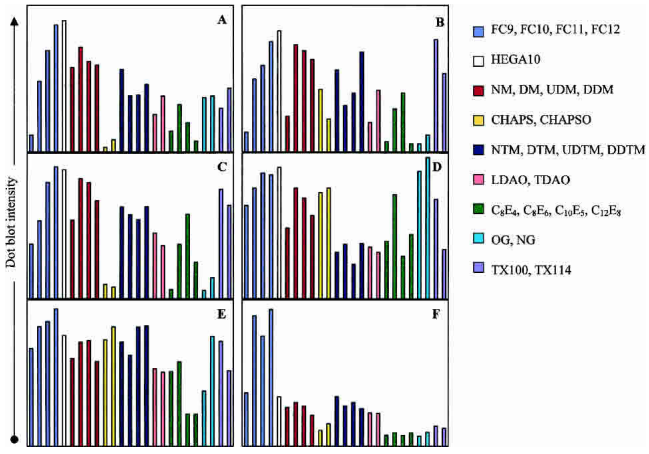

Figure 2. Dot-blot scoring of EM03 (*A*), EM04 (*B*), EM05 (*C*), EM23 (*D*), EM35 (*E*), and EM43 (*F*) from the detergent screen.

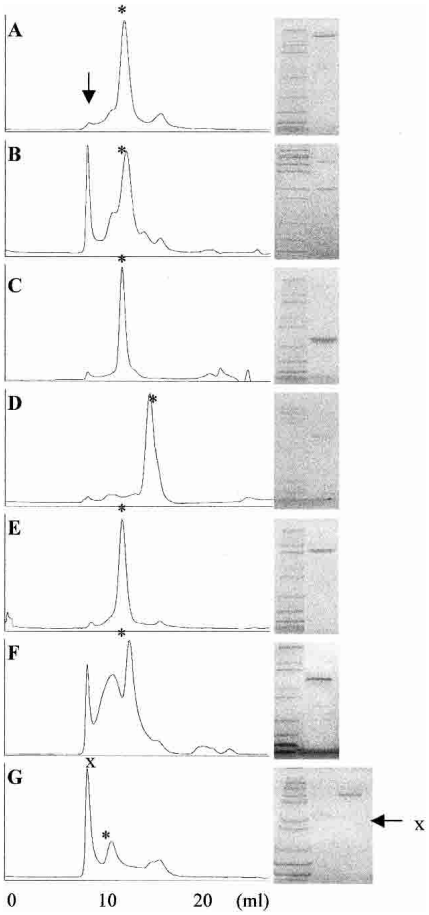

Figure 3. Gel filtration and SDS-PAGE analysis: (*A*) EM03, (*B*) EM04, (*C*) EM05, (*D*) EM23, (*E*) EM35, (*F*) EM43, and (*G*) EM47 ("X" indicates unknown protein present in the void fraction). The arrow indicates void. The asterisks indicate the fractions used to determine the yield. The proteins were FLAG-tagged, expressed in C41, and finally purified with either DDM (*A–D,G*) or FC12 (*E,F*). Purification of EM03, EM05, and EM47 with FC12 gave the same results as *A,C,G*.

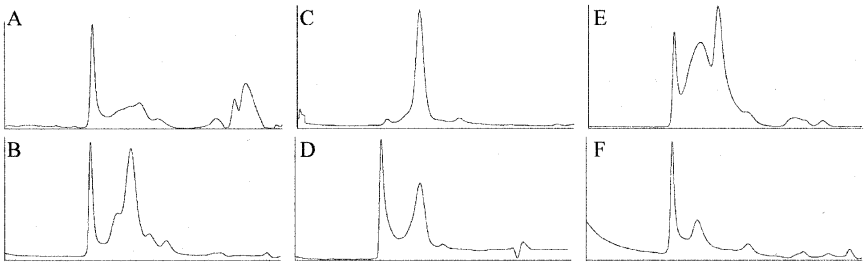

Figure 4. Comparison of detergent effect on some proteins, using gel filtration. EM04 purified with FC12 (*A*) and DDM (*B*). EM35 purified with FC12 (*C*) and DDM (*D*). EM43 purified with FC12 (*E*) and DDM (*F*).

efficient one in extracting proteins at high yield. Altogether, the presented platform provides a high success rate. The latter is especially important, since some of the proteins might have been classified as low expressed or no expression, if some traditionally used detergents, such as CHAPS, octyl glucoside, or Triton X-100, were used.

Our scale-up study shows a good correlation between the small-scale and large-scale experiments, suggesting that it will be possible to produce most of these proteins in milligram amounts. The behavior of these proteins during gel filtration indicates that most of them are indeed in a stable and well-folded form. Based on the gel filtration results from presented work and others (Auer et al. 2001), it is very important to choose the right detergent in order to preserve the integrity of each protein. The addition of the mini-scale His-tag purification step was beneficial for a number of reasons. First, we were able to distinguish between solubilized membrane proteins and vesicles, since the affinity of vesicles and membrane fragments to Ni-NTA agarose resin is extremely low (data not shown). Second, the expressed proteins may aggregate after extraction from the membrane, depending on the nature of the protein and the detergent used. Also, extensive delipidation has been reported to cause protein aggregation and precipitation (Auer et al. 2001; Boulter and Wang 2001; Lemieux et al. 2002). However, the precipitates cannot efficiently bind to the Ni-NTA agarose resin, and either pass through or bind to the filter. Finally, by determining the amount of purified material, a good indication of the amount of culture required for large-scale protein production needed for crystallization studies may be obtained. Standard methods for detergent screening, involving membrane preparation and ultracentrifugation, are not only time consuming, but also expensive due to the high cost of detergents. In addition, performing such a screen in a HTP manner involving a large number of detergents and proteins is not feasible. Here, we have screened 25 detergents from nine different families against 12 proteins, a total of 300 different conditions, in the course of a few hours, starting from cell lysis. Therefore, introducing the 96-well 6-His-tag purification method dramatically increases the throughput in screening solubilizing detergents of integral membrane proteins. Moreover, the method is cost effective and requires only standard laboratory equipment and handling; it can also be automated.

Since membrane proteins are idiosyncratic in their interactions with detergents, it is impossible to identify the 'best' detergents a priori. Ideally, a detergent (or detergent mixture) should extract and solubilize the target protein from the membrane and also have a stabilizing effect to prevent the protein from forming aggregates. In a recent report, ~20 detergents were screened in crystallization set-ups (Chang and Roth 2001). Indeed, the next rate-limiting step in membrane protein crystallography is finding the detergent suitable for crystallization screens. However, it is quite likely that the detergents that keep the protein most stable are among the best suited for crystallization. Thus, a useful subset of detergents could be identified at an early point using the presented detergent screen. Once a few detergents have been selected, other HTP approaches are needed to discard those causing larger and heterogeneous oligomers (work in progress). Thus far, we have performed large-scale purification of a dozen membrane proteins and all, except for EM43, have been successfully purified to homogeneity, of which one has resulted in diffracting crystals (data not shown).

In conclusion, the presented study demonstrates that an efficient generic HTP methodology can be implemented for the production of native bacterial integral membrane proteins. The success rate is significantly higher than anticipated and in fact comparable to the success rates of producing soluble bacterial proteins, although the production levels are lower. In order to implement HTP technology for structural studies on for example human membrane proteins, it is important to evaluate such technologies for the bacterial counterparts. We believe the presented work is the first step toward this goal.

Materials and methods

Detergents

Triton X-100 and Triton X-114 were purchased from ICN Biochemicals (Labora). All other detergents were purchased from Anatrace.

Recombination cloning of genes using Gateway technology

Coding sequences for the proteins on the target list were obtained from SwissProt database and used for primer design using SGD Webprimer design program.

Genes encoding proteins EM01–EM49 were amplified by PCR. Touchdown PCR was performed using Platinum Pfx DNA polymerase (Invitrogen); primers containing at least 15 gene-specific nucleotides (TAG Copenhagen, http://www.tagc.com); and the *E. coli* template K12λ isolated with the High Pure PCR Template Preparation Kit (Roche).

Another PCR was performed using the same Pfx polymerase, primers containing *att*B sites (Invitrogen), and the PCR products from the touchdown reactions as templates. The products were purified using the QIAquick Purification Kit (Qiagen, VWR International).

The linear fragments flanked by *att*B sequences were subjected to site-specific recombination with pDONR201 vector (Invitrogen), containing the *ccd*B gene, flanked by *att*P sites and catalyzed by BP Clonase (see manufacturer's protocols) yielding entry clones that were used to transform *E. coli* competent DH5α cells. Transformants were grown on LB agar (LA) plates (LabMedicine) containing 50 μg/mL kanamycin. Colonies were picked from each plate for colony PCR (using *Taq* polymerase and outer pDONR primers (Invitrogen)) and growth in liquid culture for subsequent plasmid preparation. Plasmid constructs were isolated using the QIAprep Spin Miniprep Kit (Qiagen).

The entry clones were subjected to another round of site-specific recombination catalyzed by the LR Clonase enzyme mix (Invitrogen) in order to subclone the genes of interest into a number of destination vectors (AstraZeneca) containing the *ccd*B gene flanked by *att*R sites, as well as the coding sequences for fusion tags (6-His, FLAG, and MBP), to generate expression clones (see Invitrogen' protocols).

The resulting expression constructs were used to transform *E. coli* C41(DE3) (C41) (Avidis, Saint-Beauzire), C43(DE3) (C43) (Avidis), and BL21(DE3) (BL21) (Novagen) strains. Transformants were selected on LA plates containing 30 μg/mL tetracycline. Growth of transformants was performed as described above. Identity of all constructs was verified by dideoxy sequencing.

Protein overexpression

The cells were grown in either shake flasks or 96-deep-well plates at 37°C until the cultures reached the OD_{600} of ~0.8. The cultures were then cooled down to 18°C and induced overnight with 0.1 mM isopropyl-β-D-1-thiogalactoside (IPTG). Cells from 1 mL fractions were harvested by centrifugation in 96-deep-well plates and finally stored at −80°C. For gel filtration analysis, 250 mL cultures were grown and induced as described above and the cells were harvested and stored at −80°C. The typical final OD_{600} was between 2 and 2.5.

Cell lysis and membrane protein extraction and purification in 96-well plates

The frozen cell pellets (obtained from 1 mL of culture) in 96-deep-well plates were resuspended in 50 μL 20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1 mg/mL lysozyme, complete protease inhibitor cocktail EDTA-free (Roche), 10 U/mL benzonase (VWR International), and 1%–2% detergent according to Table 3 (extraction). Lysis and extraction were performed for 1 h at 4°C. The suspension was filtered using a 96-well 0.45 filter plate (Millipore). The filtrate was added to Ni-NTA agarose resin (Qiagen) pre-equilibrated with purification buffer (P-buffer) containing 20 mM Tris-HCl at pH 8.0, 300 mM NaCl, 5 mM β-mercaptoethanol, and the appropriate detergent at a concentration above its critical micelle concentration (CMC) (Table 3). After 15 min of agitation at 4°C, the unbound material was removed by 30 sec of centrifugation at 100*g*. The resin was then washed with 40 mM of imidazole in the P-buffer containing the appropriate detergent at 100*g* for 30 sec. The bound recombinant membrane proteins were finally recovered in P-buffer containing 500 mM of imidazole and the respective detergent, and centrifugation at 100*g* for 1 min.

Medium-scale protein purification and gel filtration

Cell pellets from 250 mL cultures were thawed, resuspended in 20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol, and 1 mg/mL lysozyme, sonicated and centrifuged at 15,000*g*. The membranes were finally harvested by 1 h centrifugation at 150,000*g*. The membranes were resuspended and solubilized with the detergent, as stated elsewhere, in the P-buffer supplemented with 20 mM of imidazole using a glass homogenizer, followed by centrifugation for 45 min at 200,000*g*. The clear supernatants containing solubilized membrane proteins were loaded on Ni-NTA agarose resin pre-equilibrated with the P-buffer, including 20 mM of imidazole and the corresponding detergent. The resin was washed with the P-buffer containing 40 mM of imidazole and the corresponding detergent. The recombinant proteins were eluted with 500 mM of imidazole in the same buffer.

The integrity of purified membrane proteins was confirmed by gel filtration using Superdex 200 column (Amersham Biosciences). The yields were calculated by measuring the absorbance at 280 nm.

Dot-blot analysis

Of the sample, 1.5 μL was applied to nitrocellulose and allowed to dry. The 6-His-tagged proteins were detected using INDIA His-Probe-HRP Western blotting probe (Pierce) according to the manufacturer's protocol. The signals were detected with FluorS-multiImager (BioRad) and quantified using the Quantity One software (BioRad).

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