Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 327 (2005) 650–655
BBRC
www.elsevier.com/locate/ybbrc

Forceful large-scale expression of "problematic" membrane proteins☆

Ekaterina I. Mokhonova[1], Vladislav V. Mokhonov[1], Hiroyuki Akama, Taiji Nakae*

Department of Molecular Life Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara 259-1193, Japan

Received 6 December 2004
Available online 21 December 2004

Abstract

We developed an *Escherichia coli* expression system for overproduction of a highly toxic membrane protein that is impossible to overexpress by traditionally used approaches. The method is based on combination of the genetic modifications of a bicistronic expression plasmid, stabilization of a synthesized protein, and selection of a compatible expression host. This enabled us to enhance the expression level of a toxic membrane protein 30–50 times compared with expression in the native state and to obtain 3–5 mg of a highly purified functionally active protein per liter of culture. We describe the method for the amplified expression of membrane proteins, using the *Pseudomonas aeruginosa* multidrug resistance protein, MexY, as an example. The amplified MexY was correctly folded in the cytoplasmic membrane of the *E. coli* without forming inclusion bodies. This method can be applicable to the large-scale expression of the other problematic membrane proteins that are otherwise extremely difficult to overproduce.

Keywords: Large-scale expression; Xenobiotic transporter; Membrane protein; Bicistronic plasmid; Efflux pump; Expression vector

It was estimated that at least 50% of all drug targets are membrane proteins. However, they represent only 0.25% of structures in the Protein Data Bank, reflecting the numerous difficulties in their structural investigations. The principal cause is problematic overexpression of membrane proteins in high yields. Owing to the extreme hydrophobicity, membrane proteins are refractory to direct manipulation and can be removed from the membrane and maintained in solution only in the presence of a surfactant. To allow the atomic level of structural study of membrane transport proteins, a continuous supply of milligram quantities of a protein is required, whereas native expression levels are usually less than 0.1% of the total cellular proteins [1].

The *Pseudomonas aeruginosa* xenobiotic transport protein, MexY, provides a model system for analysis of structure and function of the RND-family proteins. Structural and functional characterization of this important aminoglycoside extrusion machinery is significantly restricted due to very low yield of this protein from natural sources (less than 0.1 mg/L of culture) and its high toxicity to the *P. aeruginosa* and *Escherichia coli* cells (Nakae et al., unpublished data). The strategy of amplified expression of some membrane proteins [1] was largely unsuccessful for overproduction of MexY (Mokhonov, unpublished data). The sections below describe the procedure of high-level expression of the MexY protein as an example of a "problematic" membrane protein.

☆ *Abbreviations:* DDM, *n*-dodecyl-β-D-maltoside; IPTG, isopropyl β-D-thiogalactoside; RND, resistance-nodulation-cell division; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; MDR, multidrug resistance; RBS, ribosome-binding signal; SD, Shine–Dalgarno.

* Corresponding author. Fax: +81 463 93 5437.
E-mail address: nakae@is.icc.u-tokai.ac.jp (T. Nakae).
[1] Present address: Department of Microbiology and Parasitology, School of Molecular and Microbial Sciences, The University of Queensland, Australia.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.12.059

Materials and methods

Strains, plasmids, and primers. Bacterial strains and plasmids used in this study are listed in Table 1. Primer sequences are given in Table 2. Oligonucleotides used were designed and assessed using the DNA-STAR 5.0 software package and Amplify 1.2 to minimize non-specific DNA amplification.

Construction of pMexY^{his} and bicistronic pMexXY^{his} plasmids. To construct *mexY* with histidine tag into pET22b+, the 3143 bp fragment of *mexY* was amplified using pfu-Turbo DNA polymerase (Stratagene) with primers MexYFNdeIand MexYRXhoIusing genomic DNA of *P. aeruginosa* PAO4090 as template. The PCR product was gel-purified using Qiaquick gel extraction kit (Qiagen), digested by *Nde*I and *Xho*I, and cloned into *Nde*I–*Xho*I-restricted pET22b+. The plasmid encoded MexY containing 10 histidine residues at the carboxyl terminal end was designated as pMexY^{his} (Table 1).

The bicistronic expression plasmid, pMexXMexY^{his}, was created in several steps. First, the following fragments were obtained using the PCR technique: MexX with the *E. coli* AcrA leader sequence and whole MexY with the hexa-histidine tag. The AcrA leader sequence is needed to ensure the effective and correct translocation of MexX to the inner membrane of *E. coli* cells. MexX with the AcrA leader sequence was amplified from a plasmid that was constructed previously with primers AcrAleaderF and MexX*Eco*RI (Table 2). MexY with the 3′ terminal end encoding a sequence six extra histidine residues was amplified using *P. aeruginosa* (PAO4090 strain) as the template with appropriate pairs of primers. Moreover, we used two-round PCR for insertion of the upstream start codon of the canonical Shine–Dalgarno sequence of MexY. All fragments were ligated into the pBluescriptII SK+ vector into the blunt *Eco*RV site yielding two intermediate constructs, pBS-AcrAleaderMexX and pBS-MexY^{his}.

Fragment MexY from pBS-MexY^{his} was obtained by treating *Eco*RI and *Bam*HI. This fragment was ligated with *Eco*RI and *Bam*HI-linearized plasmid pBS-AcrAleaderMexX, yielding pBS-AcrAleaderMexXMexY. The final bicistronic expression plasmid, pMexXY^{his}, was created by treating pBS-AcrAleaderMexXMexY with *Nde*I, *Bam*HI and the ligated AcrAleaderMexXMexY cassette into pET11a was digested with Nde*I* and *Bam*HI. The final expression plasmid was designated as pMexXY^{his}, where 17 and 9 nucleotides of non-coding spacers were inserted between the stop codon of MexX, and RBS of MexY and between RBS and the start codon of MexY, respectively (Fig. 2). We found that Ala, which stabilizes the proteins expressed in *E. coli* [2], was located next to the 1st methionine codon of our target protein.

MexY expression and purification. Escherichia coli C43(DE3) [2] cells were transformed with pMexXY^{his} and selected on 2× YT medium [1.6% (w/v) tryptone (Difco), 1.0% (w/v) yeast extract (Difco), 0.5% (w/v) NaCl, and 2.0% (w/v) agar (Difco), pH 7.2] plates

Table 1
Strains and plasmids

Strains or plasmids	Description	Source
Escherichia coli		
Top10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr* BC) ϕ80*lac*Δ M15 Δ*lac*X74*deo*R *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
BL21(DE3)	F$^-$ *ompT hsd*SB (rB$^-$mB$^-$) *gal dcm*(DE3)	Novagen
C43(DE3)	BL21(DE3) derivative with uncharacterized mutations	Avidis
C41(DE3)	BL21(DE3) derivative with uncharacterized mutations	Avidis
Pseudomonas aeruginosa		
PAO4290	*leu-10*, *argF10*, *aph-9004*; FP	Matsumoto collection
Plasmids		
pET11a	Expression vector Ap^r	Novagen
pET22b+	Expression vector Ap^r	Novagen
pBluescriptII SK+	*E. coli* cloning vector Ap^R	Stratagene
pMexY^{his}	pET22b+ derivative with MexY gene	This work
pMexXY^{his}	pET11a derivative with synthetic operon carrying MexX and MexY^{his} gene	This work

Table 2
Oligonucleotides used for the plasmid construction and sequencing

Name	Sequence 5′–3′	Template
T7 promoter	CGCGAAATTAATACGACTCACTAT	—
T7 terminator	GTTATGCTAGTTATTGCTCAGCGG	—
M13 forward	CGCCAGGGTTTTCCCAGTCACGAC	—
M13 reverse	TCACACAGGAAACAGCTATGAC	—
AcrAleaderF	TACATATGAACAAAAACAGAGGGTTTAC	—[a]
AcrAleaderR	CTAAGCTTCTTCGCATCCTGTTAGGGCTAAGCTGC	—[a]
MexX*Hin*dIII	AGAAGCTTCGGACGCCGGGAAGACTGCG	—[b]
MexX*Eco*RI	GTGAATTCTCACTGGCCCGCCGGCGAGGC	—[b]
MexY1F*Eco*RI	GAAGGAGAGCTACGAATGGCTCGTTTCTTC	—[b]
MexY2F*Eco*RI	GAGAATTCTTTAACTTTAAGAAGGAGAGCTACGAATGGC	—[b]
MexY1R*Bam*HI	TGGTGGTGGGCTTGCTCCGTGGGGATCTG	—[b]
MexY2R*Bam*HI^{his}	CCGGATCCTCAGTGGTGGTGGTGGTGGTGGGC	—[b]

[a] Genomic DNA of *E. coli* (Top10).
[b] Genomic DNA of *P. aeruginosa* (PAO4290).