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Coupling site-directed mutagenesis with high-level expression: large scale production of mutant porins from *E. coli*

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Abstract

Combination of an origin repair mutagenesis system with a new *mutS* host strain increased the efficiency of mutagenesis from 46% to 75% mutant clones. Overexpression with the T7 expression system afforded large quantities of proteins from mutant strains. A series of *E. coli* B^E host strains devoid of major outer membrane proteins was constructed, facilitating the purification of mutant porins to homogeneity. This allowed preparation of 149 porin mutants in *E. coli* used in detailed explorations of the structure and function of this membrane protein to high resolution.

Keywords: *E. coli* porin; Gene inactivation; High-level expression; Outer membrane protein; Site-directed mutagenesis

1. Introduction

Porins from *Escherichia coli* outer membranes form water-filled, voltage-gated channels allowing the diffusion of nutrients and waste products [1]. Using X-ray analysis, we have studied the structure of a non-specific porin, OmpF [2], and a porin specific for the transport of maltose and maltooligosaccharides, maltoporin (LamB) [3,4] to atomic resolution. Their functional properties were investigated by single channel conductance measurements and by liposome swelling assays [5]. The two porins have a remarkably similar overall architecture although they do not exhibit significant sequence homology. To establish their molecular pathology, we initially used half a dozen of naturally selected mutations [6,7]. An extensive exploration of structure-function relationships of a channel protein requires, however, a systematic mutagenization program. We have therefore developed and optimized a site-directed mutagenesis and expression system using the following criteria. (i) The mutagenesis protocol should be efficient, simple, reproducible, and avoid second site mutations and multiple subcloning steps. (ii) An expression system should provide high protein yields yet allow fine-tuning of expression levels in case an overexpression of mutant proteins has toxic effects. (iii) The least possible amounts of other outer membrane proteins should be expressed by the host cells,

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facilitating rapid purification of the desired gene product.

A modification of an origin repair mutagenesis [8] was devised to satisfy these requirements, and a universal host strain for porin gene expression by deleting all genes encoding major outer membrane proteins was constructed. Combining these approaches, 94 OmpF and 55 LamB mutant proteins were expressed which are now investigated by high-resolution X-ray analysis, liposome swelling, and single channel recording.

2. Materials and methods

2.1. Bacteriological and genetic techniques

E. coli strains, bacteriophages, and plasmids used are listed in Table 1. Cells were grown at 37°C on 2×YT medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl/l), with addition of antibiotics (ampicillin (Ap) 100 μg/ml, chloramphenicol (Cm) 30 μg/ml, kanamycin (Km) 50 μg/ml, tetracycline (Tc) 10 μg/ml) where required. P1 transduction was carried out as described [9]. To select against recipient cells, transduction mixtures were grown to an OD_{600nm} 0.2 and infected with bacteriophages (multiplicity of infection of 1) which use the protein to be inactivated as a receptor. From Tc^R transductants, Tc^S clones were selected [10].

2.2. Recombinant DNA methods

Standard DNA manipulations were performed [11] and routine DNA isolation was carried out using commercial kits (Qiagen). For Southern blot analysis, DNA fragments of *E. coli* genes coding for outer membrane proteins were labeled radioactively with [α-^{32}P]-dATP (Amersham), using a Random Primer DNA labeling kit (Bio-Rad). The fragments used were *Eco*RV-*Nde*I for *lamB* (543 bp; pAC1); *Hin*cII-*Bgl*II for *ompF* (900 bp; pMY222); *Eco*RI-*Eco*RV for *ompC* (554 bp; pMY150); and *Eco*RV-*Pst*I for *ompA* (1244 bp; pRD87). Chromosomal DNA from various *E. coli* strains was purified by Qiagen tip-100G columns and digested with restriction endonucleases. After agarose gel electrophoresis (0.8% w/v), DNA was transferred to Hybond-N membranes and hybridized with the labeled gene probes (overnight at 65°C) as recommended by the manufacturer (Amersham).

2.3. Construction of expression plasmids

As a starting point, *phoE* expression plasmid pGPhoE was constructed. A 1108-bp *Ssp*I-*Nsi*I fragment from pJP29, containing the *phoE* gene was cloned into plasmid pGEM-MAZf(+) using its filled-in *Xho*I and *Pst*I sites. From this plasmid, the 1155-bp *Xba*I-*Bam*HI *phoE* fragment was subcloned into the expression vector pET-3a. The complete *phoE* expression cassette, including an upstream T7 promoter and a downstream T7 terminator, was then transferred as a 1349-bp filled-in *Bgl*II-*Eco*RV fragment to the *Pvu*II-opened pGEM-5Zf(+), thus adding the f1 origin of replication to the expression cassette and leading to pGPhoE. For construction of the *ompF* expression plasmid pGOmpF (Fig. 1), the 1026-bp *Pst*I-*Sma*I *phoE* fragment of pGPhoE was replaced by the 1196-bp *Pst*I-*Nla*IV *ompF* fragment from pMY222. Both the *phoE* and *ompF* genes have an identically positioned *Pst*I site at the region which encodes the processing site of their signal peptides. Therefore, a chimeric protein is synthesized from pGOmpF consisting of the PhoE signal peptide and the full-length mature OmpF protein. The *lamB* expression plasmid pGLamB (Fig. 1) was constructed similarly and carries a 1519-bp *Sal*I-*Stu*I *lamB* fragment, originating from pAC1, between the T7 expression control signals.

2.4. Site-specific mutagenesis

Site-directed mutagenesis using origin repair [8] was adapted from the Altered Sites mutagenesis kit (Promega). First, a template plasmid with a defective origin of replication was produced. Such plasmids can only propagate in specialized host strains with a mutated *rnhA* gene, such as AK101. To this end, a two base-pair deletion was introduced into the *ori* regions of the plasmids pGOmpF and pGLamB by replacing their 1371-bp *Afl*III-*Sca*I fragments with the corresponding fragment from plasmid pTZ18Urrh. The resulting plasmids, pGOmpFrrh and pGLamBrrh, were transformed into *E. coli* AK101, and the R408 helper phage was used for

isolation of single-stranded DNA. Oligonucleotides used as mutagenic primers or for origin repair were 5′-phosphorylated in One-Phor-All *PLUS* buffer (Pharmacia) using T4 polynucleotide kinase and 1 mM ATP. Annealing of oligonucleotides to the single-stranded templates, second strand polymerisation by T4 DNA polymerase, and ligation by T4 DNA ligase (both from New England Biolabs) were performed as single-tube reaction (Promega). Routinely, 5 μl of the reaction volumes were used

Table 1
E. coli strains, bacteriophages, and plasmids used in this study

Strain, bacteriophage, or plasmid	Relevant genotype or phenotype	Source
E.coli K-12		
AK101	*rnhA*::*cat* derivative of JM101 (*supE thi* Δ(*lac-proAB*) [F′ *traD*36 *proAB laqI*^{Q}*Z*ΔM15])	H. Ohmori [8]
BMH71-18 *mutS*	*supE thi* Δ(*lac-proAB*) *mutS*::Tn*10* [F′ *proAB laqI*^{Q}*Z*ΔM15]	Promega
BRE2413	BRE51, Δ*ompA* linked to Tn*10*	E. Bremer [19]
DH5α	F^{-}, Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(r_K^- m_K^+) *phoA supE*44 λ^{-} *thi*-1 *gyrA*96 *relA*1 [φ80d*lacZ*ΔM15]	Gibco BRL
DH5α *mutS*	DH5α, *mutS*::Tn*10*	This study
RAM191	Δ*ompC178* linked to Tn*10*	S. Benson [20]
E. coli B^{E}		
BL21(DE3)	F^{-}, *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
BL21(DE3)omp1	BL21(DE3), Δ*lamB*	This study
BL21(DE3)omp2	BL21(DE3), *ompF*::Tn*5*	This study
BL21(DE3)omp3	BL21(DE3), Δ*lamB ompF*::Tn*5*	This study
BL21(DE3)omp4	BL21(DE3), Δ*lamB ompF*::Tn*5* Δ*ompA*	This study
BL21(DE3)omp5	BL21(DE3), Δ*lamB ompR*	This study
BL21(DE3)omp6	BL21(DE3), Δ*lamB* Δ*ompC*	This study
BL21(DE3)omp7	BL21(DE3), Δ*lamB ompF*::Tn*5* Δ*ompC*	This study
BL21(DE3)omp8	BL21(DE3), Δ*lamB ompF*::Tn*5* Δ*ompA* Δ*ompC*	This study
BZB1107	*ompR* Δ*lamB ompF*::Tn*5*	This laboratory [7]
Bacteriophages		
K3	OmpA-specific bacteriophage	U. Henning
K20	OmpF-specific bacteriophage	S. Benson
λ	LamB-specific bacteriophage	Our lab collection
Ox2	OmpA-specific bacteriophage	U. Henning
R408	Helper phage for ssDNA isolation	Promega
SS4	OmpC-specific bacteriophage	T. Silhavy
Plasmids		
pAC1	pBR322, *lamB*	M. Hofnung [21]
pET-3a	*E. coli* T7 system expression vector, Ap^{R}	Novagen
pGEM-5Zf(+)	*E. coli* cloning vector, Ap^{R}	Promega
pGEM-MAZf(+)	pGEM-7Zf(+) (Promega) with a *Nde*I-*Pst*I-*Nco*I linker insertion between the *Eco*RI and *Kpn*I sites	A. Prilipov, M. Schneider
pGLamB	pGEM-5Zf(+)-pET-3a hybrid plasmid, *lamB*	This study
pGLamBrrh	pGLamB with defective *ori*	This study
pGOmpF	pGEM-5Zf(+)-pET-3a hybrid plasmid, *ompF*	This study
pGOmpFrrh	pGOmpF with defective *ori*	This study
pGPhoE	pGEM-5Zf(+)-pET3a hybrid plasmid, *phoE*	This study
pJP29	pACYC184, *phoE*	J. Tommassen [22]
pMY150	pBR322, *ompC*	M. Inouye [23]
pMY222	pBR322, *ompF*	M. Inouye [24]
pRD87	pUC8, *ompA*	U. Henning [25]
pTZ18Urrh	pTZ18U (Bio-Rad) derivative with defective *ori*	H. Ohmori [8]

for transformation of DH5α *mutS* competent cells. All mutations were confirmed by DNA sequencing, using a commercial T7 Sequencing Kit (Pharmacia). Primer design was very simple; for one codon change, nine complementary bases bracketing the exchanged nucleotides were included.

2.5. Rapid isolation of outer membrane proteins and purification

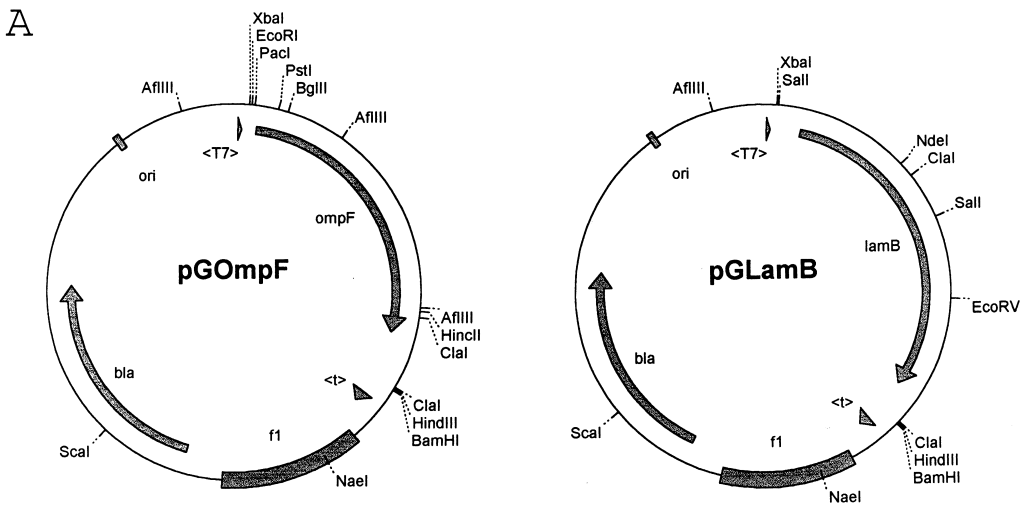
Overnight cultures (1.5 ml), pelleted in a benchtop microcentrifuge for 5 min, were washed with 1.5 ml of 10 mM $MgCl_2$ (pH 7.3), resuspended in 1.5 ml of the same solution and sonicated in an ice bath (1 min, Branson sonifier, model 250). Unbroken cells were removed by centrifugation at 5000 rpm (Eppendorf, 2 min). Recovered supernatants were centrifuged for an additional 30 min at top speed. Pellets were solubilized in 20 μl sample buffer and analysed by SDS-PAGE [12].

E. coli strains BL21(DE3)omp8 and BL21(DE3)omp5 were used to overexpress OmpF and LamB variants, respectively. Purification of porins was performed as described, with minor modifications [13,14].

3. Results

3.1. Construction of expression plasmids and site-directed mutagenesis

High-copy number expression vectors were constructed for the *ompF* and *lamB* genes (see Section 2). The respective plasmids pGOmpF and pGLamB (bold in Table 1; Fig. 1) carry an Ap resistance gene,



B

pGOmpF: *TAATACGACTCACTATA*GGGAGACCACAACGGTTTCCCTCTAGACTCGA**ATTCGCGCGTTAATTAA**
XbaI *EcoRI* *PacI*

pGLamB: *TAATACGACTCACTATA*GGGAGACCACAACGGTTTCCCTCTAGA**GTCGACTGCATAAGGAGCCG**
XbaI *SalI*

Fig. 1. Physical maps of porin expression plasmids. A: pGOmpF (left), pGLamB (right). Coding regions (bla, ompF, lamB), phage T7-specific promoter (⟨T7⟩) and terminator (⟨t⟩) are shown, as is the origin of replication of the plasmid (ori) and phage f1 (f1). B: The sequences at the fusion regions between the T7 promoter of plasmid pET-3a and the phoE (pGOmpF, upper sequence) or lamB (pGLamB, lower sequence). The T7 promoter is shown in italics, the transcriptional start sites are indicated by asterisks. Relevant restriction sites are underlined. Sequences of the structural genes of the porins are shown in bold; the translational initiation codons are located 133 nucleotides (pGOmpF) and 147 nucleotides (pGLamB) downstream of the transcriptional start sites.

a f1 origin of replication, and the gene of interest as placed under the control of a T7-specific promoter in front of a T7 terminator.

The efficiency of site-directed mutagenesis [8] was significantly improved by using a mismatch repair-deficient *mutS* strain for DNA transformation. With strain BMH28-71 *mutS*, the efficiency of transformation was so low that in some experiments no transformants were obtained at all. The *mutS*::Tn*10* mutation was therefore transduced into strain DH5α to yield strain DH5α *mutS* (bold in Table 1) which had an efficiency of transformation approaching that of the parental strain. Using this strain yielded a high number of mutants which could be screened directly by DNA sequencing. A single mutagenic primer yielded 75% mutated clones; while using two or more mutagenic primers simultaneously resulted in a lower, but satisfactory fraction of mutants. The successful application of this method to construct numerous *ompF* and *lamB* mutants is illustrated in Table 2. Five mutations could thus be introduced at different sites using two (LamB) and four (OmpF) mutagenic primers simultaneously (see asterisks in Table 2).

3.2. Construction of various BL21(DE3) derivatives

In order to allow large amounts of mutant porins to be purified, a universal expression host lacking the major outer membrane proteins LamB, OmpA, OmpC, OmpF was constructed. A series of mutants of BL21(DE3) with defects in the *lamB*, *ompA*, *ompC*, *ompF*, and/or *ompR* genes was engineered by P1 transduction of known mutations from other strains. Strains BZB1107 (*ompR*, Δ*lamB*, *ompF*::Tn*5*), BRE2413 (Δ*ompA*), and RAM191 (Δ*ompC*) served as donors. Transductants were first selected by resistance to phages which use the protein to be inactivated as a receptor (Table 1). All mutant BL21(DE3) derivatives (Table 1) were then assayed for lack of the corresponding proteins by SDS-PAGE analysis (Fig. 2). Finally, displacement

Table 2
Porin mutants generated by site-specific mutagenesis

OmpF	LamB
Single mutations	
K16A, K16C, **V18K**, **H21T** [26], K25A, K25C, M38C, **R42A**, E48A, E48Q, **E71A**, **E71Q**, **D74A**, **D74N**, **R82A**, F96C, F96R, **R100A**, **R100S**, **Y102F**, **Y106F**, **D113C** [27], **D113N**, M114C, **E117C** [15], **E117Q** [27], S125C, **S125H**, D127N, D127V, D127T, G131K, **R132A**, Y139F, F144R, F145R, F153Q, Y157F, Y180R, E181A, E181Q, Y182R, Y185R, I187E, G189E, Y191R, D195C, W214R, Y220R, Y231R, Q255C, Y263R, F265R, F267R, **S272A** [15], S272C, Y275E, F295Q, **E296A** [15], **E296Q** [15], **E296L** [15], **E296M**, Y301R, **D312N** [15], D312V, D312T, Y313R, S320R, **A333C** [15]	**Y6A**, Y6N, **R8A**, **R33A**, **Y41A**, Y41R, **E43A**, E43Q, N63C, **W74A**, W74L, **R82A**, S98C, **R109A**, **D111A**, **H113A**, H113Q, **D116A**, F117A, **Y118A**, Y119A, W120A, R175C, T216C, **F227A**, F227I, F230A, T233S, M291C, S321C, T336C, N341C, **W358A**, W358H, T368C, T412C, **W420A**, W420K
Double mutations	
V18K/G131K, M38C/M114C, M38C/S125C, M38C/D195C, M38C/Q255C, R42A/R82A, R42A/R132A, **E71A/R100A**, **E71A/R100S**, **E71Q/R100A**, **E71Q/R100S**, R82A/R132A, D113N/E117Q, M114C/D195C, M114C/Q255C, **E117C/A333C** [15], S125C/D195C, S125C/Q255C, D195C/Q255C	**Y6A/R8A**, **Y6A/W420A**, **R8A/R33A**, **R33A/E43A**, **R33A/H113A**, **R33A/D116A**, **Y41A/W420A**, E43A/K45D, **E43Q/R109A**, **R82A/D116A**, **R109A/D111A**
Multiple mutations	
R42A/R82A/R132A, R42A/D113N/E117Q, **R42A/R82A/D113N/E117Q/R132A***	**Y6A/R8A/Y41A**, **Y6A/Y41A/W420A**, **R109A/H113A/D116A**, **R109A/Y118A/D121Y**, **R33A/E43A/R109A/H113A/D116A***
Deletion mutations	
Δ105-112, Δ105-129, Δ116-120 [15], Δ118-133	Δ107-110

Mutants indicated in bold have been characterized functionally, structurally, or both (see references in parentheses) or their study is in progress. Asterisks indicate quintuple mutants obtained by the use of more than one mutagenic primer simultaneously (see text).

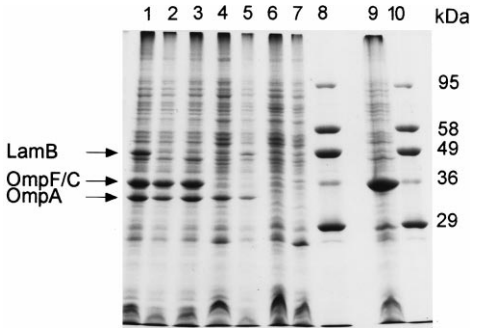


Fig. 2. Protein profiles of membrane extracts analysed by SDS-polyacrylamide gel electrophoresis. The various derivatives of BL21(DE3) are shown, with the position of migration of the major outer membrane proteins (LamB, OmpF, OmpC, and OmpA) indicated on the left. Lane 1 shows the parent strain. Lanes 2–10 display the patterns of the various deletions in the structural or the regulatory genes of the porins (cf. Table 1). Key: lane 1, BL21(DE3); lane 2, BL21(DE3)omp1; lane 3, BL21(DE3)omp6; lane 4, BL21(DE3)omp5; lane 5, BL21(DE3)omp3; lane 6, BL21(DE3)omp4; lane 7, BL21(DE3)omp8; lane 9, BL21(DE3)-omp8, which carries a deletion in the chromosomal *ompF* gene, but overexpresses it from pGOmpF plasmid; lanes 8 and 10, molecular weight marker.

of porin genes (*lamB*, *ompF*, *ompC*) and *ompA* was confirmed by Southern blot analyses (data not shown).

3.3. Expression of OmpF and LamB in BL21(DE3) derivatives

For the overexpression of porins, the corresponding expression plasmids, pGOmpF and pGLamB, were transformed into porin-deficient BL21(DE3) mutant strains. When OmpF was expressed in BL21(DE3)omp8, it was found to produce the most abundant amounts (Fig. 2). Numerous mutant OmpF proteins were purified to homogeneity. Heat modifiability, detected by SDS-PAGE, indicated a trimeric state. The resulting crystals diffracted X-rays to 2.2–3.1 Å [5,15]. Analogous results (not shown) were obtained with LamB, using BL21(DE3)omp5 as the expression host.

4. Discussion

We have devised a system that combines a simple and rapid method of mutagenesis with adjustable expression levels. For high-level expression, we used the well-established T7 RNA polymerase/promoter system [16]. The *E. coli* B^E strain BL21(DE3), lysogenic for bacteriophage DE3 and therefore allowing IPTG-induced overexpression of T7-controlled genes [16], has the advantage that it lacks the OmpT outer membrane protease and does not express the OmpC porin [17]. In order to simplify purification of plasmid-encoded mutant porins, all chromosomal genes encoding major outer membrane proteins (*lamB*, *ompA*, *ompC*, *ompF*) were inactivated in this strain. Alternatively, a mutation in the regulatory *ompR* gene was introduced, which also resulted in the repression of OmpF and OmpC synthesis. Despite the lack of major pore-forming proteins, the strains grew rather well due, possibly, to the expression of the product of a cryptic porin gene. When provided with the corresponding expression plasmids, production of OmpF or LamB-porins occurred in high amounts (Fig. 2). The series of isogenic strains with non-reverting mutations in genes encoding outer membrane proteins provides the basis for a study of the interactions of these proteins also with toxins or other proteins of the bacterial cell envelope, such as the TolA-protein [18].

The origin repair system for site-directed mutagenesis [8], which is based on parental DNA strand discrimination, avoids both the presence of a second antibiotic resistance gene in the plasmid as well as second rounds of transformation. Second site mutations, often associated with PCR-based mutageneses, have not been observed. Moreover, since we performed both mutagenesis and expression experiments with the same plasmid, it was also not necessary to subclone mutated genes into expression vectors. The efficiency of mutagenesis of 46% mutant clones, observed by Ohmori [8], compares to an average efficiency of mutagenesis of 75%, achieved here by the introduction of the mismatch repair deficient strain DH5α *mutS*. In comparison to similarly efficient PCR-based systems, the need for purification of single-stranded DNA is the most serious disadvantage of the method presented here. In cases where the large numbers of mutants in one or few genes are critical for an in-depth characterization of their products, the high efficiency outweighs this drawback, while the conventional procedure may be more convenient if small numbers of mutations are

sought in many different genes. So far, our approach yielded 149 mutants. The coupled mutagenesis-expression system described should, of course, be readily applicable to other porins also, such as the OmpC-protein and phosphoporin, allowing the construction of many more porin mutants for functional and structural characterization.

Acknowledgments

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