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Use of the Gram-negative Bacterial ABC Transporter System in Biotechnology: Enhancement of Secretory Production of Recombinant Proteins in *Escherichia coli*

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Gram-negative bacteria use several strategies to secrete proteins across the inner and outer membranes into the extracellular environment. So far, five classes of secretion pathways have been identified in these bacteria [1]. The ATP-binding cassette (ABC) transporters are an important class of transport proteins, and the ABC-dependent type I secretion systems are also widespread in gram-negative bacteria. One of the ABC pathways of gram-negative bacteria is unique because it is able to transport large polypeptides across both the inner and outer membranes into the extracellular surroundings [2, 3].

Proteins secreted by the gram-negative bacterial type I pathway lack an amino-terminal (N-terminal) signal sequence. They cross both membranes without a periplasmic intermediate. The target proteins of these ABC-mediated polypeptide exporters, such as proteases [4], lipases [5], toxins [6] and S-layer proteins [7], have an uncleaved carboxy-terminal (C-terminal) signal sequence containing several repeats of the glycine-rich sequence (GGXGXD) and a predicted amphipathic α-helix region. ABC transporter consists of three components, the ATP Binding Cassette (ABC) protein, the Membrane Fusion Protein (MFP), and the Outer Membrane Protein (OMP). ABC protein is an inner membrane protein composed of an N-terminal membrane domain containing six to eight transmembrane segments and a C-terminal ATPase domain. ABC protein belongs to the well-characterized ABC protein superfamily which includes eukaryotic and prokaryotic proteins related to the import or export of a wide variety of substrates, such as ions, antibiotics, sugars, amino acids, oligosaccharides, peptides and proteins [8]. It recognizes the C-terminal signal sequence of target protein and supplies energy from ATP hydrolysis for secretion of target protein [9]. MFP is exposed mainly to the periplasm and has one transmembrane segment anchored in the inner membrane. MFP connects ABC protein and OMP during formation of the transport complex. OMP is an outer membrane porin protein that forms a tunnel across the periplasm and the outer membrane [10].

In virtue of its considerable advantages, there have been many attempts to develop a strategy to promote the secretion of recombinant proteins into extracellular space [11]. One of the strategies to secrete recombinant proteins into extracellular space is the transplantation of secretion machinery into the non-pathogenic *E. coli* host and the *Salmonella* strians. Because most of the non-pathogenic *E. coli* strains

secrete few proteins to the extracellular space, the recombinant secretion machinery for protein production has usually originated from other bacterial species as well as from pathogenic *E. coli*. Among several secretion machineries, ABC transporter (Type I secretion pathway) appears to be a more attractive candidate for the secretory production of recombinant proteins in *E. coli* and some other bacteria. ABC transporter, in particular the HlyB-HlyD-TolC translocator of *E. coli* to secrete the hemolysin A, has received some biotechnological attention as a system for production of recombinant proteins in *E. coli*. First, it is important to increase the efficiency of secretion of the target protein (for example, by improving the ability of ABC transporter to secrete). In addition, increasing the amount of the functional ABC transporter located in the cellular membrane can be another way to increase the efficiency of secretion of a target protein.

Extracellular production system of recombinant proteins in *E. coli*

Some factors – including the target specificity of ABC transporter, the total amount of ABC transporter found in the membranes, and the expression rate of target protein – are regarded to be important experimental parameters, which can influence the efficient secretion of target foreign protein by heterologous ABC transporter in recombinant *E. coli* host. Among the above-listed factors, we examined the effects of both the expression amount of ABC transporter and target protein and the relative expression ratio between them. To control the expression level of *tliA* (i.e., the *Pseudomonas fluorescens* lipase secreted by the TliDEF transporter) and *tliDEF* (i.e., the *P. fluorescens* ABC transporter secreting the TliA lipase) in *E. coli* [12], we constructed several *tliA-tliDEF* co-expression systems using two replicon-compatible vectors, pKK223-3 which is a medium copy number plasmid under the control of the IPTG-inducible tac promoter and pACYC184 which is a low copy number plasmid under the control of the weak constitutive tet promoter. Three gene regions, *tliDEF*A, *tliDEF* and *tliA*, were amplified by PCR and subcloned to make the six resultant plasmids (Table 1).

We determined the time course profiles of cell growths and TliA secretion levels for the recombinant *E. coli* cells which harbored four *tliA-tliDEF* co-expression systems at the concentration of 0.01 mM IPTG optimized previousely. While *E. coli* (pTliDEFA-223+pACYC184) showed the highest TliA secretion level, *E. coli* (pTliDEF-223+pTliA-184) and *E. coli* (pKK223-3+pTliDEFA-184) showed the lowest TliA secretion level. It meant the optimization of the expression levels and the relative expression ratio of target protein and ABC transporter contributed to the increase of target protein secretion level. Moreover, the IPTG induction timing was found to have little effect on the cell growth since the O.D.600nm values of

Table 1. Co-expression systems of *tliA* and *tliDEF* in *E. coli*.

Co-expressed recombinant plasmids	pKK223-3-based genes	pACYC184-based genes
pTliDEFA-223 + pACYC184	*tliDEFA*	-
pTliA-223 + pTliDEF-184	*tliA*	*tliDEF*
pTliDEF-223 + pTliA-184	*tliDEF*	*tliA*
pKK223-3 + pTliDEFA-184	-	*tliDEFA*

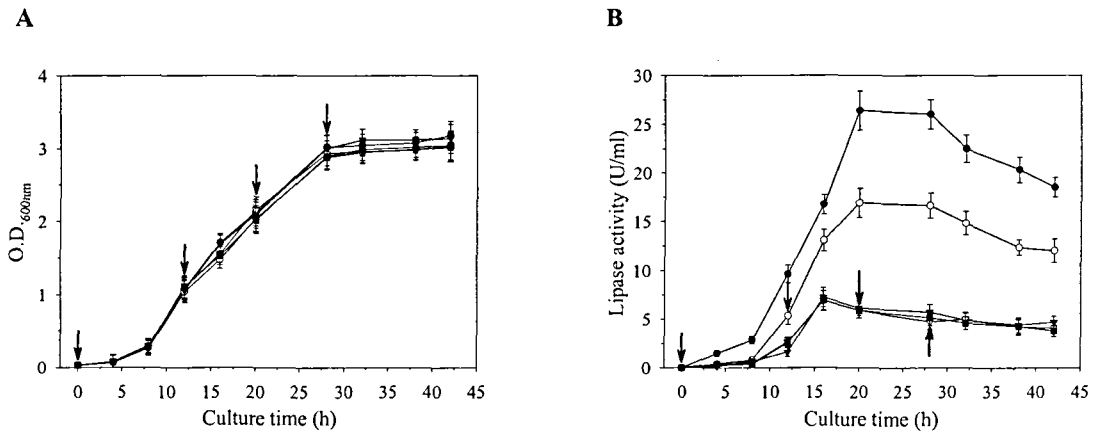

Fig. 1 Cultivation of *E. coli* cells containing each *tliA-tliDEF* co-expression system.

each cell were almost same during the culture time (Fig. 1A). However, IPTG induction timing showed a significant effect on the TliA secretion level (Fig. 1B). As the induction timing was earlier, the TliA secretion level of the recombinant cell increased. When IPTG was induced at O.D.600nm values of 2.0 and 3.0, it did not increase the TliA secretion level. The TliA secretion level of *E. coli* (pTliDEFA-223 + pACYC184) was highest (27 U/ml) when IPTG was added to the cell culture at time zero. When IPTG was induced at the early phase of cell growth, the number of TliA which could interact with TliDEF and the number of TliDEF anchored in the cellular membrane would simultaneously increase as the culture time increased. Thus, the cell induced at the early growth phase would secrete more TliA compared to the cell induced at the middle or late growth phase at the same culture time, resulting in the higher TliA secretion level.

Mutagenesis of the ABC protein-encoding gene

To make ABC transporter a more efficient production system for recombinant proteins, it is essential to enhance its secretion efficiency. We applied directed evolution techniques (error-prone PCR) to create TliD variants and isolated some variants exhibiting the increased secretion level of the heterologous TliA lipase in *E. coli* (Fig 2). The ABC transporter (TliDEF) from *P. fluorescens* SIK W1, which mediated the secretion of a thermostable lipase (TliA) into the extracellular space in *E. coli*, was engineered using directed evolution (error-prone PCR) to improve its secretion efficiency. TliD mutants with increased secretion efficiency were identified by co-expressing the mutated *tliD* library with the wild-type tliA lipase in *E. coli*, and by screening the library with a tributyrin-emulsified indicator plate assay and a microtiter plate-based assay. Some selected mutants from one round of error-prone PCR mutagenesis showed the increases of the secretion level of TliA lipase, respectively, but had almost the same expression level of TliD in membrane as that of the wild-type TliDEF transporter. These results indicated that the improved secretion of TliA lipase was mediated by these transporter mutations. Each mutant had a single amino acid change within the predicted cytoplasmic regions in the membrane domain of TliD, implying that the corresponding region of

TliD was important for the improved and successful secretion of target protein. The secretion efficiency of heterologous protein in *E. coli* could be enhanced in vitro engineering of ABC transporter.

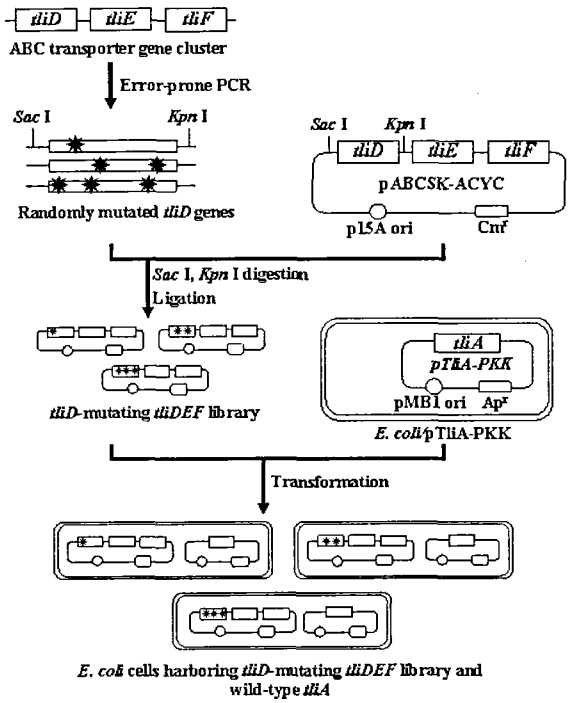

Fig. 2 Schematic representation of the procedures for constructing a library of ABC transporter mutants and coexpressing the library with the target lipase gene.

Heterologous ABC transporter as a screening system in *E. coli*

To demonstrate the application of heterologous ABC transporter to the cloning of a target protein, we constructed a bacterial genomic DNA library coexpressing an ABC transporter in *E. coli*. Two extracellular protease genes were obtained from a library member coexpressing the heterologous ABC secretion apparatus. This approach could be an alternative way to clone ABC transporter-driven hydrolytic enzymes, in particular lipases and proteases, from their hyper-producing bacteria. In order to clone the genes encoding non-cognate extracellular protein from a bacterial strain, we here employed the transporter system in *E. coli*. The genomic DNA library of a gram-negative bacterium was constructed by coexpressing with the gene cluster encoding the protein exporter (Fig 3). A positive clone was isolated on activity-indicater agar plates and revealed three open reading frames. The amino acid sequences deduced showed significant similarity to known members of gram-negative bacterial protein that had been secreted by the type I (ABC transporter-mediated) pathway. When the cloned protease was coexpressed with another ABC transporter from other bacteria, it also exhibited a sign of extracellular activity.

Our results implied that the heterologous ABC transporters can be used as a coexpression system to effectively screen the type I secretion pathway-related proteins from bacterial genetic resources. Our

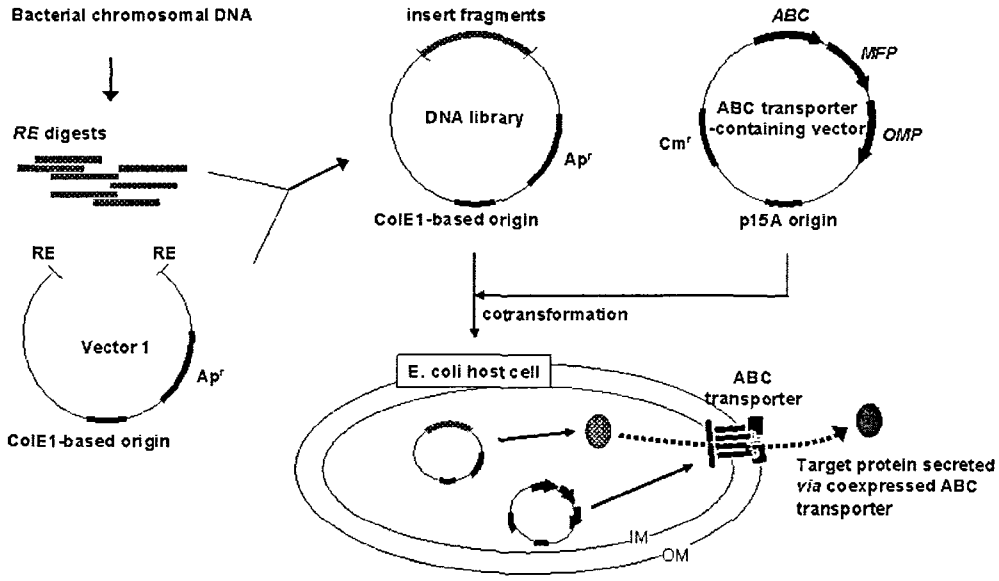

Fig. 3 Strategy for cloning of gram-negative bacterial proteins secreted via the heterologous ABC transporter-mediated secretion system in *E. coli*.

approach of using the heterologous ABC secretion system in *E. coli* proved the feasibility for more easily obtaining a number of genes encoding ABC transporter-related extracellular proteins from other microorganisms. We are now attempting to expand this strategy into the more complex environmental DNA library comprised of the genetic materials of entire microbial communities, so-called metagenome, as well as into some other gram-negative bacteria.

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