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Biotechnological applications of periplasmic expression in *E. coli*

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Introduction

Expression of recombinant proteins outside the cytoplasm was pioneered over 20 years ago in bacterial and yeast systems [1-3], opening the way for its current use in a variety of biotechnological applications [4]. The bacterium *Escherichia coli* remains the preferred host for the extracytoplasmic expression of recombinant proteins because of its compartmentalized cell envelope, which comprises the cytoplasmic and outer membrane separated by the periplasmic space [5]. Production of recombinant proteins in the periplasm offers in some cases important advantages over their cytoplasmic expression because it improves protein folding, reduces proteolytic degradation and facilitates purification [6]. Additionally, many (potential) substrates are able to cross the outer membrane unlike the cytoplasmic membrane. The increased substrate accessibility offered by periplasmic expression is a major advantage that is exploited in library screening and whole-cell biocatalysis [7]. Here, I will discuss recent progress in the periplasmic expression of recombinant proteins in *E. coli* with a focus on its use in library screening, protein engineering and whole-cell biocatalysis.

For secretion of proteins, *E. coli* possesses two distinct translocation systems, SecYEG and Tat (Figure 1) [8,9]. The vast majority of secretory proteins are translocated by the SecYEG system in an unfolded state[8], whereas the Tat system only accepts fully folded and often cofactor-containing proteins for periplasmic export [9]. The export of foreign proteins to the periplasm of *E. coli* is accomplished by harnessing endogenous protein translocation pathways, which is typically achieved by fusing a signal sequence to the target protein. The type of signal sequence used determines which export pathway is utilized by the target protein. Most Sec-dependent secretory proteins contain a SecB-dependent signal sequence and are translocated by the ATPase SecA post-translationally, while kept in an unfolded state by chaperones such as SecB or DnaK [8]. In addition, a sub-set of secretory proteins are exported in a co-translational fashion via the SRP pathway which is able, unlike the SecA/B pathway, to export fast-folding proteins, or proteins that aggregate in the cytoplasm [10-13]. Moreover, the Sec system cannot be used for the export of cofactor containing target proteins unlike the Tat system. Interestingly, a recent study suggested that Tat-dependent export results in relatively pure and highly active recombinant proteins compared to the same proteins exported Sec-dependently [14].

In summary, the choice of export pathway is dictated by the properties of the target protein. This is exemplified by our study on exporting AldO, a flavoprotein oxidase, to the periplasm of *E. coli* [15]. AldO contains covalently bound FAD, which will only form upon linkage to polypeptide chain. In contrast; AldO could be functionally transported to the periplasm Tat-dependently. Similarly, it was found that green fluorescent protein, which folds rapidly in the cytoplasm, could be transported in a functional form to the periplasm via the Tat pathway and not Sec-dependently [16,17].

Library screening and protein engineering

Periplasmic expression of protein libraries is commonly employed in protein engineering experiments to enable screening for variants displaying the desired trait. Several elegant studies show that secretory pathway quality control can be utilized in library screening. The Sec system is able to prevent the export of folded proteins and this form of quality control was exploited in the selection of a novel superfolder GFP [18]. It was found that certain variants were rejected by the Sec system and accumulated in the cytoplasm of *E. coli* as a consequence of their improved folding kinetics. The Tat translocase also exhibits secretory pathway quality control, but in this case, unfolded proteins are rejected for export. This feature of the Tat pathway was utilized to obtain solubility-enhanced variants of Alzheimer's Aβ42 peptide [19,20]. These studies emphasize the potential of secretory pathway quality control in library screening to ensure that properly folded variants are obtained.

In addition, periplasmic expression is employed in the screening of antibody libraries using cell sorting techniques. Firstly, it was shown that full length antibodies can be exported to the periplasm of *E. coli* by fusing to a SecB-dependent signal sequence. Following secretion into the periplasm, antibodies are captured by a Protein A derivative of *Staphylococcus aureus*, which is anchored to the periplasmic face of the cytoplasmic membrane [21,22]. Another system, utilizes a lipoprotein-type signal sequence for export of antibodies to the periplasm and subsequent tethering to the cytoplasmic membrane [23-25]. Both systems allow the cell sorting based screening of *E. coli* spheroplasts that display antibodies at their cytoplasmic membrane. Secondly, solubility-enhanced antibody fragments were selected from a library of antibody fragments using Tat-mediated quality control in combination with a cell sorting-based screen [26].

The increased substrate accessibility offered by periplasmic expression was recently explored by us in the development of a novel screening procedure for Baeyer-Villiger-monooxygenases (BVMOs) (our unpublished results). BVMOs are NADPH-dependent flavoproteins that catalyze a variety of oxidations [27]. To meet the demand of a generic screen for BVMOs, we based the screening procedure on Tat-mediated periplasmic export of BVMOs which allows conversion of compounds that do not enter the cytoplasm. Moreover, periplasmic expression of BVMOs enables coenzyme recycling using our established coenzyme regeneration system [28,29]. Moreover, the use of periplasmic expression was recently extended to the production of recombinant glycoproteins. *E. coli* expressing the *Campylobacter jejunipgI* gene cluster, which encodes a bacterial pathway for N-linked

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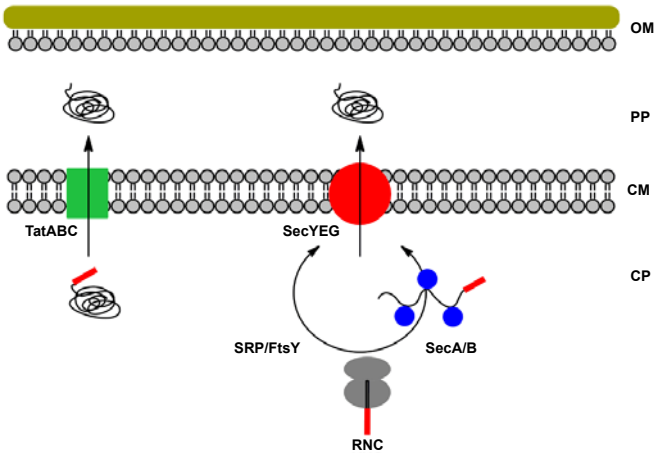

RNC: Ribosome-nascent chain complex; CP: cytoplasm; CM: cytoplasmic membrane; PP: periplasm; OM: outer membrane.

Figure 1: Biotechnologically relevant periplasmic export pathways in *E. coli*. All secretory proteins are initially synthesized on cytoplasmic ribosomes with a cleavable N-terminal signal sequence. The signal sequence funnels its passenger protein into a dedicated protein translocation pathway (see text for further details). Signal sequences are represented by red bars and molecular chaperones are indicated in blue.

protein glycosylation [30], was used to study N-linked glycosylation of secretory proteins. It was found that proteins secreted via the SecA/B, SRP or Tat pathway were successfully N-glycosylated and, moreover, a clear difference in the glycosylation pattern of these proteins was observed depending on their mode of export [31]. The secretion of bacterial glycoproteins seems, therefore, a promising tool in the production and engineering of recombinant glycoproteins.

Whole-cell biocatalysis

Whole-cell biocatalysis prevents the costly and laborious isolation of enzymes and the use of periplasmic expression of target enzymes offer a greatly improved substrate accessibility. The latter is well illustrated by our finding that cells which express AldO in the cytoplasm are unable to convert xylitol. In contrast, xylitol was readily converted by cells that export AldO Tat-dependently to the periplasm [15]. Next, we constructed a xylitol biosensor comprising AldO equipped with a microperoxidase (MP) domain. In this hybrid, both domains act in concert because the MP domain enables the detection and quantification of the oxidase activity. This eliminates the need of adding peroxidase, which is typically required for the detection of oxidase activity. Cells secreting the AldO-MP hybrid protein Tat-dependently to the periplasm were successfully used for the *in vivo* detection of oxidase activity [32].

In addition, periplasmic expression was used in several studies to develop and optimize tools for the bioremediation of organophosphates. These compounds are known to be neurotoxic and are used for the synthesis of pesticides, herbicides and nerve gasses. Organophosphates can be degraded by several bacterial enzymes such as, organophosphorus hydrolase (OPH) and methyl parathion hydrolase (MPH). Several whole-cell biocatalytic systems revolving around OPH were developed relying either on Sec-dependent or Tat-dependent export. This resulted in different biocatalytic systems capable of efficient degradation of organophosphates [33,34]. Moreover, the Tat pathway was used for the periplasmic export of MPH in *E. coli* and the biocatalytic performance of this system can be greatly improved by coexpression of chaperones [35,36].

Other platforms for protein export

Although secretion outside of the cytoplasm can in many cases be achieved by genetically fusing a signal sequence to the target protein, this strategy does, however, not always work. Several studies show that this can frequently be solved by attachment of a larger proteinaceous moiety that functions as an export signal, or secretion partner. For example, it was found that a truncated derivative of protein A is able to facilitate the periplasmic export of several recombinant proteins attached to its C-terminus, thereby enabling their facile purification from periplasmic extracts [37]. Moreover, *E. coli* OsmY was identified in a proteomic study as a potential partner for extracellular secretion and was found to facilitate the highly efficient secretion of recombinant proteins into the growth medium [38,39]. This also allows their rapid, one-step isolation.

Conclusions and perspective

Since the first landmark studies over 20 years ago, periplasmic expression of recombinant proteins in *E. coli* has become indispensable in many biotechnological applications. The latter is exemplified by only a selection of relevant studies in this editorial paper. Despite recent progress, several important challenges remain. These include the periplasmic expression of sizable, cofactor-containing proteins, coexpression of more than one target protein in the periplasm and improvement of secretion capacity. The periplasmic expression of complex (containing multiple cofactors) and large proteins remains problematic primarily because the *E. coli* Tat system is unable to handle large proteins. This is in contrast to the Tat translocon of *Streptomyces coelicolor* which is able to translocate proteins up to 146 kDa [40]. The coexpression of several proteins in the periplasm is importantin, for example, the design of cascade reactions. However, it is questionable whether this can be achieved by using different expressions systems employing different translocation pathways without avoiding detrimental secretion stress. Alternatively, the genetic fusion of relevant proteins or catalytic domains followed by periplasmic secretion via the same export route seems to be a more promising strategy as illustrated by our oxidase-microperoxidase hybrid [32]. Strategies for increasing the secretion capacity include coexpression of chaperones, translocon components and the optimization of signal sequences [41]. It can be expected that the growing understanding of protein translocation systems in combination with the increasing amount of structural data will provide new leads for the optimization of protein secretion in *E. coli*, which will ultimately extend the biotechnical use of periplasmic expression further.

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