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Biotechnological applications of bacterial protein secretion: from therapeutics to biofuel production

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Abstract

Recent years have witnessed significant progresses in engineering of recombinant protein secretion. The relatively simple secretion mechanisms, Type I and Type V (autotransporters), are increasingly used for secretion of recombinant proteins. The secretion level of target proteins varied from milligrams to grams per liter. The range of proteins was significantly expanded beyond medical application. Notable additions include biofuel productions from renewable feedstock. Despite the progress, almost all successes in the engineering efforts come with significant trials and errors, highlighting the need for a better understanding of secretion systems and rational based methods.

Keywords: Recombinant protein production; Signal peptide; Type I secretion system; Type III secretion system; Autotransporter

1. Introduction

Bacteria are increasingly used for the production of industrially relevant proteins. The ease with which bacteria accept and express new genes allows for the use of these cells in a multitude of applications. Particularly remarkable is the impact of recombinant proteins on biopharmaceutical applications. Many therapeutic proteins such as insulin, interleukin-2 and human growth hormone are produced using bacterial hosts (de Oliveira et al., 1999; Kamionka, 2011; Roifman et al., 1985). *Escherichia coli*, in particular, has been the host for the expression of viral proteins and virus-like particles for use in vaccine development (Liew et al., 2010; Middelberg et al., 2011). Besides medical applications, recombinant lipoproteins produced from *Bacillus* and *Pseudomonas* have been used in oil recovery, soil washing and bioremediation (Banat et al., 2010). One important area of application with increasing interests is biofuel production from renewable resources, where microbial cellulases and hemicellulases play essential roles in the deconstruction of polymeric lignocellulosic biomass to fermentable sugars. Accordingly, enzymes from cellulosic bacteria such as *Clostridium* and *Saccharophagus* strains and xylanases from *Klebsiella* and *Lysinibacillus* have been recombinantly produced. These enzymes are useful either as catalysts in the enzymatic hydrolysis of lignocelluloses or as components incorporated in engineered microbes for consolidated bioprocessing of lignocelluloses (Alves-Prado et al., 2010; Park et al., 2011; Peralta-Yahya et al., 2012; Sekar et al., 2012; Shin et al., 2010). In the case that whole microbial cells expressing recombinant proteins are used to interact with polymeric material, such as lignocelluloses, extracellular secretion of proteins is necessary due to the inability of microbial cells to uptake polymer substrates. In the case of applications where purified recombinant proteins are used directly, secretion of these proteins extracellularly could significantly reduce the complexity of a production process by eliminating the need for cell lysis and reducing the burden of removing host proteins. In addition, secretion of highly expressed proteins minimizes formation of inclusion bodies, aids in folding, allows for disulfide bond

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formation, reduces the effects of intracellular protein degradation and lessens the detrimental effects of cytotoxic proteins (Dalbey et al., 2012; Daniels et al., 2010; Ni and Chen, 2009).

These many potential benefits have motivated researchers to seek effective ways to engineer host cells for high level secretion of recombinant proteins. Recent advances in this regard are significant and excellent reviews on specific or general aspects of secretion can be found in literature (Blight and Holland, 1994; Clérico et al., 2008; Galán and Collmer, 1999; Jong et al., 2010; Ni and Chen, 2009). Here, we review more recent progress. We divide the review into two sections. The first section focuses on the secretion across the cytoplasmic membrane, in which we emphasize work related to Sec and Tat pathways and associated signal peptide selection and modification, as well as supplementation of culture media to promote protein secretion. In the second section, we review progresses toward secretion across the outer membrane by highlighting efforts of engineering three dedicated secretion mechanisms, Type I, III, and V secretion systems.

2. Secretion across the cytoplasmic membrane

Sec and Tat are two common pathways that allow translocation of proteins across cytoplasmic membrane in both gram-negative and gram-positive bacteria (Natale et al., 2008). The Sec pathway involves translocation of an unfolded protein across the cytoplasmic membrane, whether by co-translational or post-translational targeting. Co-translationally targeted secretion involves binding of the signal recognition particle (SRP) to an N-terminal signal sequence of the secreted protein as its mRNA is translated, whereas post-translationally targeted proteins are kept unfolded by the chaperone protein SecB. The protein is then transferred to the Sec translocase complex (SecYEG) for translocation across the membrane. Tat-dependent systems, in contrast, transport fully folded proteins across the membrane and are mediated by the Tat translocase complex (TatABC). Transport of fully folded proteins is necessary if cofactor binding is required for protein functionality. Processing of a protein by either of these pathways is mediated by its signal sequence, which contains information for recognition by the SRP and translocation machinery.

By a simple technique known as gene fusion, a signal sequence can be added to the target gene at the N-terminus of a target gene, to label the encoded protein for secretion by either pathway. Researchers have shown that a suitable signal peptide is one of the most important factors that influence secretion efficiency (Yoon et al., 2010). A common approach is to use a signal sequence from the expression host. This approach was successfully used in *Zymomonas mobilis* to express two cellulolytic enzymes from *Acidothermus cellulolyticus*, resulting in cells capable of growth on carboxymethyl cellulose (Linger et al., 2010; Mazzoli et al., 2011). This endogenous signal peptide fusion approach was also used in engineering *Bacillus subtilis*. In this case, the secretion signal of SacB (a levansucrase) from *B. subtilis* has been used to allow secretion of the *Clostridium cellulovorans* mini-cellulosome by three separate strains of *B. subtilis* into extracellular space (Arai et al., 2007). As mentioned previously, proteins targeted for co-translational secretion are processed through the SRP-dependent pathway. If the target gene is subject to high levels of transcription and its corresponding protein undergoes no post-translational modification such as cofactor binding, secretion may be improved by retargeting through the SRP-dependent pathway. For example, Steiner et al. improved phage display of designed ankyrin-repeat proteins over 700-fold by switching from a SecB to SRP-dependent signal peptide (Steiner et al., 2006).

While these approaches are widely used, there are no guiding principles in the selection of an endogenous signal sequence which will give an optimal level of a target heterologous protein. Often, success requires a laborious screening process to identify a suitable signal sequence. For example, Matos et al. have screened five endogenous *E. coli* signal peptides fused to the model fluorescent protein GFP and discovered that the Tat-dependent pathway enables more efficient secretion to the periplasm than the Sec-dependent pathway (Matos et al., 2012). Apparently, transport of folded GFP through Tat pathway is more effective than transporting unfolded GFP via Sec pathway. Signal peptides from microorganisms other than the expression host could also be used. These signal peptides are referred as exogenous peptides in this review. As with endogenous peptide signals, selection of exogenous signal peptides often entail screening a large number of candidate signal sequences. Degering et al. studied the secretion of an industrially relevant protease Subtisilin BPN' in native *Bacillus* species through screening of 393 Sec-dependent signal peptides from *B. subtilis* and *Bacillus licheniformis* (Degering et al., 2010). By screening this library of signal peptides the authors were able to identify signal peptides that increase extracellular activity to over 700% of the wild type signal protein. In another work, a functional screen of 93 Sec signal peptides from *Lactobacillus plantarum* using staphylococcal nuclease as a reporter gene identified a signal peptide from an uncharacterized gene which led to double the extracellular protein activity compared to use of known signal peptides, indicating the utility of this approach (Mathiesen et al., 2009). These studies demonstrate that use of natural signal peptides, either endogenous or exogenous (heterologous), can improve secretion of a target protein, and that screening a large number of candidate peptides may be necessary for optimal production. Recent researches have also shown that sometimes using modified signal peptides could result in increased levels of heterologous protein secretion. In some cases, modification that increases the positive charge of the N-terminal sequence or net hydrophobicity of a signal peptide was found to increase levels of secretion.For example, Ismail et al. increased the net charge of the Sec-dependent L-asparaginase II signal peptide to +5 for high level secretion of a recombinant cyclodextrin glucanotransferase to the periplasm (Ismail et al., 2011). In the same study, modification of the signal peptide also alleviated problems associated with protein overexpression such as cell lysis; mutagenesis of the signal peptide resulted in a 1.7-fold enhancement of periplasmic activity and a tenfold reduction of cell lysis. In