*J. Microbiol. Biotechnol.* (2004), **14**(1), 128–133

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# Secretion Capacity Limitations of the Sec Pathway in *Escherichia coli*

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Received: July 14, 2003
Accepted: October 14, 2003

**Abstract** The secretion capacity of two *E. coli* strains (JM109 and AF1000) was evaluated through the expression of two human proinsulin fusion proteins using the translocation signal sequence from Staphylococcal protein A (SpA). Although a 7 to 11-fold difference in the expression levels was attained by the use of different promoters (*SpA* and *malK* promoters) and copy-number vectors (700 and 50 copies per cell), the maximum translocation rates for all the systems were around 140,000 amino acids $cell^{-1}$ $min^{-1}$. Moreover, the secretion capacity was found to be independent of the size of the exiting peptide and its translational rate.

**Key words:** Secretory protein, proinsulin, secretion

Protein secretion to the *Escherichia coli* periplasm or to the culture medium has several advantages over intracellular production [23, 54], one of which is simplified recovery of the gene product. Also, secretion can be a way to guarantee the N-terminal authenticity of the expressed polypeptide, since secretion often involves the cleavage of a signal sequence. Biological activity is dependent on the folding state of the protein and proper folding is unlikely to occur in the cytoplasm, particularly if disulfide bonds have to be formed, due to its reducing environment [32, 41].

Protein secretion in *E. coli* is a particularly complex process [9, 42], and attempts to secrete recombinant proteins can face several problems. Most frequently, these problems are due to the incomplete translocation across the inner membrane [2], the insufficient capacity of the export machinery [30, 43], and proteolytic degradation [17].

Several factors can influence the secretion of a recombinant protein in *E. coli*. It has been reported that the size of the passenger polypeptide may influence the secretion efficiency [21, 39, 48] and that large cytoplasmic proteins may be physically impossible to translocate [2,10]. The amino acid compositions of the leader peptide [33, 34] and of the target protein [1, 5, 19, 52, 53] are also important in protein secretion.

Secretory proteins are usually translocated through the cytoplasmic membrane in a Sec-dependent manner. Unlike inner membrane proteins, that are targeted by the Signal Recognition Particle (SRP) pathway [13, 58], secretory proteins normally require the assistance of the molecular chaperone SecB for translocation.

Secretory proteins targeted to the SecB-dependent pathway usually contain a short amino-terminal extension, the signal peptide, which acts both as targeting and recognition signal [11, 61]. Ribosome-associated nascent chains of these proteins bind a prolyl isomerase known as the trigger factor with a high affinity [14, 15, 40], which prevents their co-translational binding to SRP components [3]. SecB then interacts with the mature region of the preprotein, in order to prevent its premature folding, and targets it to the membrane-bound ATPase SecA [20, 24]. Protein translocation occurs at the SecYEG channel [7, 60] through SecA membrane cycling [4]. Multiple rounds of SecA insertion and de-insertion promote preprotein threading through the channel [9, 62]. When the substrate is halfway through the translocase, the proton-motive force alone can complete the translocation even in the absence of SecA [35, 36]. SecA expression is down-regulated by binding of SecA molecules to its own mRNA [11], a control mechanism not seen in other components of the SecB-dependent pathway [42]. SecA is also translationally regulated by an upstream gene, *secM* (previously named gene X), that has been proposed as a sensor for the secretion proficiency state of the *E. coli* cell [37]. This regulation involves repression of *secA* translation under conditions of excess protein secretion and derepression when SecM secretion becomes limiting [47].

This work reports on the existence of an upper limit for the translocation capacity of *E. coli* cells expressing recombinant human proinsulin fusion proteins, using the leader peptide from *Staphyloccocus aureus* protein A

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(SpA) for translocation. The same maximum translocation rate was obtained with two different sized proteins in two *E. coli* strains, independent of the translational level.

## MATERIALS AND METHODS

### Expression Vector Construction

The two exons of the human proinsulin gene were assembled as previously reported [28]. The fragment was cloned in the pEZZ18 vector (Pharmacia, Uppsala, Sweden) and this construction was named pFM7 [26]. This vector is a high copy-number plasmid (containing the ColE1origin of replication) for ZZ-proinsulin production under transcriptional control of the *SpA* promoter. Vector pFM17, producing Z-proinsulin, was obtained by incomplete restriction of pFM7 with *Acc*I (Promega, Madison, U.S.A.), followed by gel purification of the relevant fragment and self-ligation with T4 DNA ligase (Promega). Vector pFM16 [30] is a low copy-number vector (pMB1 origin of replication) for ZZ-proinsulin production under transcriptional control of the *malK* promoter.

### Cultivation Conditions and Growth Medium

*Escherichia coli* JM109(DE3) (Promega) was transformed with plasmids pFM7 and pFM17, whereas strain AF1000 [45] was transformed with plasmid pFM16. Cells were grown in 500-ml shake-flasks (37°C, 220 rpm) with 100 ml M9 medium containing 4 g/l glucose [44] supplemented with 100 µg/ml ampicillin (Sigma, St. Louis, U.S.A.). For the JM109 (DE3) cultures, 5 mM thiamine (Sigma) was added to the M9 medium. For the pFM16 vector (*malK* promoter), cultures were grown in 100 ml M9 medium containing 4 g/l maltose (instead of glucose).

A correlation between viable cell number and optical density (OD) of the culture (measured at 600 nm) was obtained by serial dilution plating of culture volumes at different growth stages. Three independent cultivations were used for the dilution plating experiments, resulting in the following correlation: $1OD=8.01\times10^8$ cells/ml ($r^2=0.99$).

### Protein Analysis

The periplasmic extract was prepared by an osmotic shock procedure from 1 ml cell culture as previously described [29], and analyzed by an indirect ELISA method [27]. Total expression was analyzed by densitometry of Western blotting membranes as previously described [26]. In this assay, the intensity of the bands from the culture samples is compared with the one generated by affinity purified ZZ-proinsulin standards. Due to some type of interference, this method underestimates the total expression levels (total expressed recombinant protein was apparently less than the secreted fraction assayed by ELISA). It is likely that the fusion protein originating from the cytoplasm is in inclusion bodies, whereas the standards originate from the periplasmic space where inclusion bodies are in principle not formed. Although several denaturing conditions were used to efficiently solubilize the inclusion bodies [27], the signal from the Western blotting membranes did not increase, probably due to inefficient denaturing conditions or protein chemical degradation. Therefore, the intensity of protein bands from the samples was determined by densitometry, and the results from different membranes were normalized in relation to the intensity of the bands from the standards. The final results are presented in arbitrary units per cell.

## RESULTS AND DISCUSSION

The secretion capacity of two *E. coli* strains (JM109 and AF1000) was evaluated through the expression of two fusion proteins (ZZ-proinsulin and Z-proinsulin), using the signal peptide from Staphylococcal protein A (SpA) for translocation. The growth curves of the recombinant strains show that a higher cellular density could be achieved with the AF1000 strain bearing the pFM16 plasmid, although the maximum growth rates were similar for all the systems (Fig. 1).

Due to the correct processing of the SpA signal sequence, as previously reported [26], and through *in silico* analysis (not shown), it is likely that both recombinant protein fusions are translocated by a SecB-dependent mechanism.

Plasmid pFM16 is a low copy-number vector (pMB1 derived) carrying the *malK* promoter [30]. Plasmids pFM7 [26] and pFM17 are high copy-number vectors (ColE1

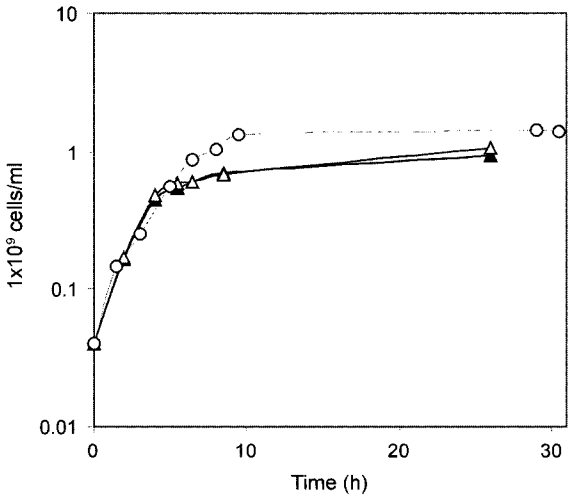


**Fig. 1.** Growth curves of recombinant cultures.
(○) AF1000 cells harboring the pFM16 vector, producing ZZ-proinsulin; (▲) JM109 cells containing the pFM7, vector producing ZZ-proinsulin; (△) JM109 cells with the pFM17 vector, producing Z-proinsulin.

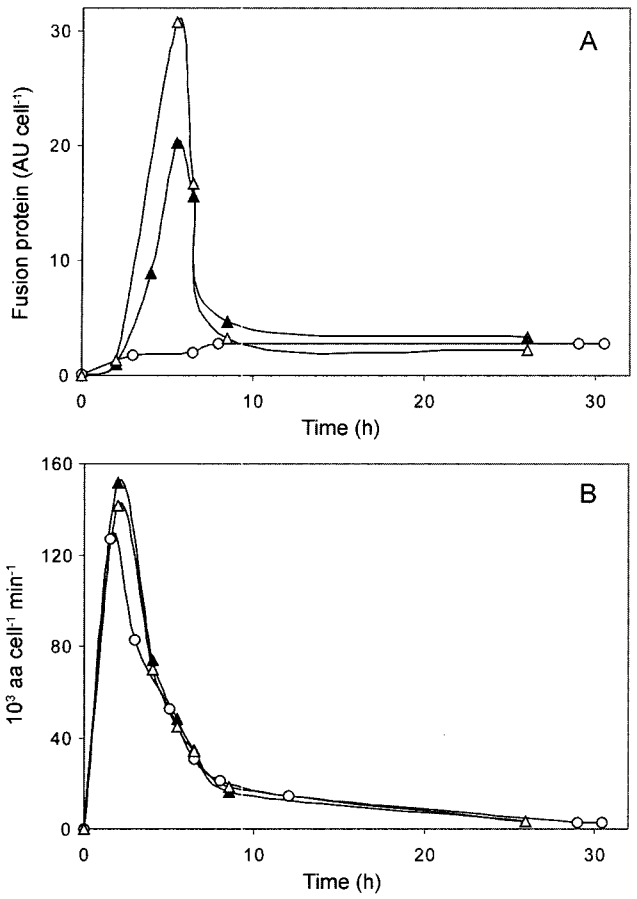


**Fig. 2.** Expression and secretion of proisulin fusion proteins in two *E. coli* strains.
A, total expression; B, periplasmic secretion. (○) pFM16 vector producing ZZ-proinsulin in AF1000 cells; (▲) pFM7 vector producing ZZ-proinsulin in JM109 cells; (△) pFM17 vector producing Z-proinsulin in JM109 cells.

based) carrying the SpA promoter. By using these two types of vectors, it was possible to attain different expression levels, with the SpA promoter systems displaying higher translational levels (7 to 11-fold) than the *malK* promoter system (Fig. 2A). Despite those differences, the maximum yield for periplasmic translocation was similar for all constructs (Fig. 2B) suggesting the existence of a secretion capacity bottleneck in the translocation of these proteins.

The maximum translocation rate occurred before the maximum expression level for all the systems (Fig. 2), and this rate was similar for all of them (around 140,000 amino acids $cell^{-1}$ $min^{-1}$). After this maximum rate is attained, the translocation efficiency sharply decreased, probably due to the agglomeration of cytoplasmic protein being synthesized at a rate that was higher than the secretion capacity of the cells.

Recently, a three-dimensional structure for the bacterial protein-translocation complex SecYEG was proposed [6], indicating that an active translocation channel is composed of dimers of SecYEG (maximum 250 per cell [42]). Accordingly, the secretion capacity of the Sec system should be around 20,000 amino acids/min.translocase, but not the previously reported value of 10,000 amino acids per minute per translocase [42].

By using two different expression levels and two different proteins, a maximum translocation value of 140,000 amino acids/cell.min (3% of the total secretion capacity) was reached in the two *E. coli* strains. Although the leader peptide is likely to play a key role in the definition of this upper limit [34, 38, 57, 61], in the present study, maximum level was independent of the protein size (Table 1). Indeed, the secretion of large multimeric proteins has been achieved with the SecB-dependent system [16], suggesting that the threading mechanism of this pathway should not impose a size limit constraint on its substrates [51]. Moreover, our results indicate that the translational level does not affect the maximum translocation capacity (Fig. 2). Nevertheless, excessive expression rates should be avoided, particularly if long production periods are required, because of the energy requirements and the sequestering of protein synthesis machinery that occurs, when a cell is overproducing a protein that it does not need [8, 49]. Therefore, a lower expression vector (like pFM16) seems to be more adequate for production purposes, since it allows a relatively high secretion level with a lower total expression (Table 1).

It has been reported [50] that an optimum translational level exists to achieve high-level secretion of heterologous proteins, otherwise secretion severely drops off. We propose that this type of limitation exists when protein is being

**Table 1.** Recombinant protein production and secretion. The number of amino acids (aa) and molecular weight of each fusion protein are indicated. The maximum values obtained with these systems regarding total expression and periplasmic secretion are given in arbitrary units per cell (AU/cell), and femtograms of recombinant protein per cell (femtogram/cell), respectively. The maximum secretion rate obtained with each system is indicated in the last column (in thousands of amino acids per cell and min).

| Vector name | Fusion partner | Size (aa) | Mol. weight (kDa) | Total[a] (AU/cell) | Periplasm[b] (femtogram/cell) | Secretion rate (1e3 aa/cell.min) |
|---|---|---|---|---|---|---|
| pFM7 | ZZ | 251 | 24.05 | 20.2 | 15.4±1.1 | 151.7 |
| pFM16 | ZZ | 251 | 24.05 | 2.8 | 11.2±2.0 | 127.2 |
| pFM17 | Z | 193 | 17.42 | 30.8 | 13.2±1.4 | 141.4 |

[a]Measured by densitometry of Western blotting membranes from a representative cultivation. The standard error for the method was lower than 12.5%.
[b]Measured by indirect ELISA. The values result from at least two independent cultivations.

synthesized at such a high rate that it agglomerates in the cytoplasm in a translocation-incompatible state, although the capacity of the cell translocases has not been overwhelmed. This type of limitation can be explained by an insufficient amount of SecB in the cytoplasmic pool (or by a non-efficient interaction between SecB and the expressed protein) with the concomitant formation of inclusion bodies. This model explains why translocation may be compromised by high translational rates, whereas a lower expression can use the full capacity of the secretion system.

The type of limitation that we describe here is related to the translocation capacity of the cells [43, 55], and should be independent of the translational rate as long as the exiting peptides remain in a translocation-competent state.

Secretion capacity bottlenecks have been previously reported by other groups [12, 31], but it is often unclear if translocation is hampered by an excessive translational rate or if the secretion systems are actually working at their full capacity. There are other cases, similar to this study, when it has been established that the secretion capacity is independent of the translational level [22], and an explanation is lacking for this phenomenon.

It has been suggested that SecA release from the membrane, concomitant with ADP release from SecA, is the rate-limiting step for translocation [24], and therefore, optimizing SecA de-insertion may be an attractive strategy to extend the export capacity of *E. coli* cells. Thus, overproduction of SecA could in principle enhance translocation by promoting the exchange between cytosolic and membrane-bound SecA, thus stimulating SecA release. Although SecA expression is regulated by SecM [37, 47], it has been reported that certain signal peptide mutations in SecM can prevent its translocation, thereby rendering SecA expression constitutive [46] which could be beneficial for recombinant protein secretion. Since a complete understanding of SecA regulation is currently unavailable, the most promising strategy to increase the translocation capacity consists of the use of *E. coli* strains carrying mutations in some of the *sec* genes (*prl* mutations) [24, 59].

The existence of a secretion capacity bottleneck has been demonstrated in *E. coli* cells. The value of this upper limit (3% of the maximum export capacity, with the SpA signal peptide) seems very low, suggesting either a saturation of the secretion system with host proteins or a reduced affinity of the recombinant protein to the Sec components. The overexpression of heterologous proteins, concomitant with the saturation of the secretion capacity, represents an imbalance in the host physiology which is usually accompanied by deleterious effects [8, 49] and the triggering of several metabolic cell alarms [18]. These effects will often limit cell growth and the duration of the production phase in a process scheme. This problem is particularly severe in *E. coli*, since most recombinant protein secretion is done by the Sec mechanism that is not translationally controlled, unlike the SRP mechanism [13, 56] which is controlled by elongation arrest [25]. Therefore, when recombinant proteins are produced in *E. coli* and targeted to secretion by the SecB-dependent pathway, the rate of recombinant protein synthesis must be optimized in order to avoid inclusion body formation. The best way to attain this control is at the transcriptional level, which can be done, for instance, by carefully balancing the promoter strength and gene copy number [27, 30], thus enabling longer production periods through a more rational use of the cell resources.

## Acknowledgments

F.J.M. Mergulhão acknowledges the receipt of a post-doctoral fellowship from the Operational Programme for Science Technology and Innovation, Ministério da Ciência e Tecnologia, Portugal. Prof. G. Larsson (Swedish Centre for Bioprocess Technology, Stockholm, Sweden) and Prof. A. Farewell (Department of General and Marine Microbiology, University of Gothenburg, Sweden) are acknowledged for providing the *malK* promoter and the AF1000 strain. S. Ribeiro (Instituto Superior Técnico, Portugal) is acknowledged for the determination of viable cell numbers.

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