Biochimica et Biophysica Acta 1808 (2011) 2544–2550

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# Directed evolution of efficient secretion in the SRP-dependent export of TolB

Yaramah M. Zalucki [a], William M. Shafer [a,b], Michael P. Jennings [c,*]

[a] Department of Microbiology and Immunology, Emory University, Atlanta, GA 30322, USA
[b] Laboratories of Bacterial Pathogenesis and Antimicrobial Resistance, VA Medical Center, Decatur, GA 30033, USA
[c] Institute for Glycomics, Griffith University, Gold Coast Campus, Gold Coast, QLD 4222, Australia

ARTICLE INFO

*Article history:*
Received 23 January 2011
Received in revised form 15 May 2011
Accepted 6 June 2011
Available online 16 June 2011

*Keywords:*
Protein export
Codon usage
Evolution
Signal recognition particle

ABSTRACT

Signal sequence non-optimal codons have been shown to be important for the folding and efficient export of maltose binding protein (MBP), a SecB dependent protein. In this study, we analysed the importance of signal sequence non-optimal codons of TolB, a signal recognition particle (SRP) dependent exported protein. The protein production levels of wild type TolB (TolB-wt) and a mutant allele of TolB in which all signal sequence non-optimal codons were changed to a synonymous optimal codon (TolB-opt), revealed that TolB-opt production was 12-fold lower than TolB-wt. This difference could not be explained by changes in mRNA levels, or plasmid copy number, which was the same in both strains. A directed evolution genetic screen was used to select for mutants in the TolB-opt signal sequence that resulted in higher levels of TolB production. Analysis of the 46 independent TolB mutants that reverted to wild type levels of expression revealed that at least four signal sequence non-optimal codons were required. These results suggest that non-optimal codons may be required for the folding and efficient export of all proteins exported via the Sec system, regardless of whether they are dependent on SecB or SRP for delivery to the inner membrane.



## 1. Introduction

Proteins targeted for translocation across the inner membrane via the general secretory pathway (Sec) contain an N-terminal signal peptide. This signal peptide has an average length of 30 amino acids, and is usually cleaved upon translocation to the periplasm by signal peptidase I [1]. The signal peptide is divided into three regions, a positively charged N-terminal region, hydrophobic core and C-terminal cleavage sequence [2]. Prior to translocation to the periplasm, the signal peptide can interact with the mature protein and modulate the folding dynamics in the cytoplasm [3–5].

Proteins exported via the Sec system are typically targeted to the inner-membrane via two alternate pathways. One is SecB, a cytosolic chaperone that directs the preprotein to SecA in the inner membrane. The other is signal recognition particle (SRP) that preferentially binds more hydrophobic signal peptides [6] and directs them to FtsY in the inner membrane.

Non-optimal codons are defined by their low usage in the genome, and a correspondingly low level of their isoacceptor tRNA [7,8]. The translation rate at these codons is slower than at the synonymous optimal codon as measured in *Escherichia coli* [9,10]. In addition, codons with inefficient codon–anticodon interactions have a slower translation rate compared to efficient interactions and can be classified as non optimal, independent of their relative frequency [11].

In a previous study, we reported that signal sequences of secreted proteins in *E. coli* have a significantly higher frequency of non-optimal codons than both the N-terminus of non-secreted proteins, and the average frequency for all coding sequences [12]. This same phenomenon has been observed in the Gram-positive bacterium *Streptomyces coelicolor* [13]. Further studies demonstrated that for maltose binding protein (MBP) [14] and β-lactamase [15], two proteins used as model systems to study protein export, changing non-optimal to optimal codons in the signal sequence lowered expression compared to the wild-type signal sequence. Production of MBP was recovered when expressed in strains deficient in multiple cytoplasmic or periplasmic proteases [14]. We hypothesised that increasing the translation rate across the signal peptide resulting from changing to all optimal codons results in a misfolded conformation of the protein, which is subsequently degraded by proteases. Biochemical analysis on the purified MBP that contained the optimised signal sequence compared to the wild-type MBP showed distinct tryptic and thermodynamic profiles, indicating that their tertiary structure was different [16]. Both MBP and β-lactamase are post-translationally exported, with MBP dependent on SecB for delivery to the inner membrane [17], whilst β-lactamase is exported post-translationally [18] in a SecB and SRP independent manner [19]. However no studies have been done to determine whether optimising the codon usage in the signal sequence would affect the export of a completely SRP-dependent protein. In this study, we examine whether TolB, an SRP-dependent exported protein [6,20,21] requires non-optimal codons for efficient export and expression.

* Corresponding author.
*E-mail address:* m.jennings@griffith.edu.au (M.P. Jennings).

0005-2736/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2011.06.004

## 2. Materials and methods

### 2.1. Strains and growth conditions

All molecular cloning work was carried out in *E. coli* Top-10 (Invitrogen, C404003). The other strains used in this study are listed in Table 1. *E. coli* was grown in water baths with shaking (200 rpm) at the following temperatures: 37 °C, 30 °C or 21 °C for room temperature experiments. Strains were grown in Luria-Bertani broth containing the appropriate antibiotic at the following concentrations: kanamycin (50 μg/ml), chloramphenicol (20 μg/ml). Strain CAG59101 was also grown with 0.2% L-arabinose. The protease mutant strains were generously donated from Carol Gross laboratory.

### 2.2. Cloning of tolB into pGBS19

Primers were designed to amplify *tolB* from strain MG1655 (CGSC# 6300) with the forward primer containing an EcoRI site and the reverse primer a PstI site (Table 2). The forward primer for the TolB-opt construct included all the non-optimal to optimal codon changes (Fig. 1A). The PCR produced was amplified using Phusion Taq (Finnzyme), digested with EcoRI and PstI and cloned into the vector pGBS19 [22] digested with the same enzymes. The ligated product was transformed into *E. coli* DH5α and putative transformants that had the entire *tolB* gene were sequenced (Agencourt Sequencing Facility), to confirm that only the desired non-optimal to optimal codon changes were incorporated.

### 2.3. Expression analysis of TolB-wt and TolB-opt

Single colonies from the appropriate strain were grown overnight in LB-kanamycin (50 μg/ml) at 37 °C and then diluted 1:100 into fresh LB-kanamycin. These cultures were grown until mid-log phase and then *tolB* expression was induced with 0.3 mM IPTG for 1 h. Cells were then pelleted for either Western, RNA and DNA analysis. The DNA analysis was done by extracting the plasmid DNA by miniprep, digesting with *EcoRI* and *PstI* and then running on a 1% agarose gel. The volume of DNA used in the digest was proportional to the $A_{600}$ reading of the cells spun down prior for the miniprep.

### 2.4. Western analysis of TolB production

Whole cell lysates were boiled in 1× sample buffer and run on a 10% SDS-PAGE gel. Protein samples were normalised for cell number, with approximately $1\times10^8$ cells run per sample. The membrane was incubated with anti-TolB, generously donated by Dr Benedetti [23], at 1:10,000 dilution overnight and developed using anti-rabbit IgG antibody (Bio-Rad, 170–6518) at 1:10,000 dilution conjugated with alkaline phosphatase.

### 2.5. Selecting for higher sodium cholate resistant clones

Using the same cloning strategy as above, a forward primer (TolB_deg, Table 2) was designed such that all combinations of non-optimal or optimal codons were possible in the final product. This PCR product was cloned into pGBS19 using the same strategy as described above. After transforming into *E. coli* DH5α via heat-shock for 45 s at 42 °C, they were grown overnight in LB-kanamycin (50 μg/ml) and the plasmid extracted via midiprep (Qiagen). This step was repeated four times and the plasmid pooled for transformation via electroporation into strain A593. To select for higher sodium cholate resistant colonies, 100 ng of TolB_deg, TolB-wt, TolB-opt and the empty vector pGBS19 were transformed via electroporation into TolB deficient strain A593. After grow-out phase of 30 min, the transformants were selected on plates supplemented with kanamycin (50 μg/ml), IPTG (0.1 mM) and sodium cholate (1.5 mg/ml). Another selection experiment was done with plates supplemented with 2 mg/ml sodium cholate. Colonies from TolB_deg transformation were mini-prepped and sequenced, and TolB production and MIC measured as described above.

**Table 1**
*E. coli* strains used in this study.

| Strain | Relevant genotype | CSGC#/reference |
|---|---|---|
| C600 | | 3004 |
| A593 | C600 *tolB* | 4924/[43] |
| MG1655 | | 6300 |
| CAG53101 | MG1655 *lon* | C. Gross lab |
| CAG53117 | MG1655 *lon, clpXP* | C. Gross lab |
| CAG53221 | MG1655 *lon*, *hslVU*, *clpXP* | C. Gross lab |
| CAG59101 | MG1655 *rpoH* + pBAD::groES/L | C. Gross lab |

**Table 2**
Primers used in this study.

| Primer name | Sequence |
|---|---|
| TolB-wt | TCAGCTGCAGGTCCAGATAAGGGAGATATGATGAAGCAGG |
| TolB-opt | TCAGCTGCAGGTCCAGATAAGGGAGATATGATGAAGCAGGCACTG CGCGTAGCATTTGGTTTTCTGATCCTGTGGGCAAGCGTTCTGCAT |
| TolB-deg | CGTCAGCTGCAGGTCCAGATAAGGGAGATATGATGAA GCAGGCACTNCGNGTAGCATTTGGNTTTCTNATHCTN TGGGCATCNGTTCTNCATGCT |
| TolB-rev | AGCAGTGAATTCCTTTGTTCAGTTGCATTTCAATGATTCC |
| TolB-F1 | ATGACCATGATTACGCCAAGC |
| TolB-R1 | CGCTGTCGATCACAATGCGGACTT |
| 16S-F | ACGGAGGGTGCAAGCGTTAATC |
| 16S-R | CCGCCTTCGCCACCGGTATTCCT |

### 2.6. RNA analysis by SQ-RTPCR

RNA was extracted from the cultures using RNA Mini-Kit (Qiagen) following the manufacturer's instructions. 1 μg of RNA was converted to cDNA using random hexamers and reverse transcriptase (Applied Biosytems). The cDNA served as the template for PCR reactions with primers specific for the 16S rRNA and the *tolB* transcript (Table 2) and was visualised by electrophoresis on a 1.8% agarose gel.

### 2.7. MIC of SDS and sodium cholate

The minimum inhibitory concentration (MIC) of strain A593 to SDS and sodium cholate was tested using 2-fold agar dilution method [24]. Briefly, the *E. coli* cultures were grown at either 37 °C or room temperature (21 °C) until mid-log phase and then induced with 0.3 mM IPTG for 1 h. After measuring the $OD_{600}$, the cells were diluted to a concentration of $10^4$ cells/μl. Then a 5 μl drop of cells was added to plates of various SDS or sodium cholate concentrations, which also contained 0.1 mM IPTG to maintain TolB production. The plates were incubated overnight at 37 °C.

### 2.8. Statistical analysis of codon distribution in the signal sequence

All possible combination of non-optimal codons in the signal sequence that could be generated from the degenerate primer were calculated and then categorised by the total number of non-optimal codons. This generated the following numbers of sequences with the respective numbers of non-optimal codons: 0 — 8, 1 — 248, 2 — 1608, 3 — 5656, 4 — 11,720, 5 — 14,568, 6 — 10,584, 7 — 4104, and 8 — 648. The numbers were calculated using probability distribution, given the respective probabilities at each position that a non-optimal codon could occur. The classification of codons as optimal or non-optimal was based on a study by Burns and Beacham [25].