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Biochemical and Biophysical Research Communications 366 (2008) 135–141
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Secretory signal sequence non-optimal codons are required for expression and export of β-lactamase

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Received 15 November 2007
Available online 11 December 2007

Abstract

In this study we altered the codon usage in the signal sequence of the *bla* gene, encoding β-lactamase in *Escherichia coli*. Changing all of the thirteen non-optimal codons to optimal lowered expression 4-fold as measured by minimum inhibitory concentration (MIC) to the β-lactam antibiotic ampicillin. The difference in ampicillin resistance was reduced at 28 °C compared to expression at 37 °C, suggesting that the optimised *bla* allele is misfolded and degraded by heat-shock regulated proteases. A screen was carried out, designed specifically to identify revertants with changes in codon usage resulting in higher MIC to ampicillin. The nine revertants revealed by this method all had optimal to non-optimal codon changes in the signal sequence. These results, and those of our previous study with maltose binding protein model system, confirm that non-optimal codons are important for expression and export of secretory proteins via both the SecB-dependent and -independent pathways.

Keywords: Protein export; Codon usage; Signal sequence

Non-optimal codons are defined by their low usage in the genome and the low abundance of their corresponding tRNA [1], and have an established role in translational pausing to allow the correct folding of proteins [2,3]. We have reported previously that non-optimal codons occur at high frequency in the signal sequence of secretory genes in *Escherichia coli* [4]. In particular, the striking abundance of non-optimal codons in the signal sequences of secretory proteins exported via the *sec*-dependent pathway relative to both their N-terminal mature sequences, and to the N-terminus of non-secretory proteins [4]. The high occurrence of non-optimal codons in the signal sequence of secretory proteins has also been observed in the Gram-positive bacterium *Streptomyces coelicolor* [5].

In a previous study, the non-optimal codons in the signal sequence of maltose binding protein (MBP) were changed to optimal codons [6], indicating that non-optimal codons were important for a time critical early event in the export of MBP. In the current study, the role of non-optimal codons in the expression and secretion of β-lactamase was investigated. Unlike MBP, β-lactamase is exported in a SecB-independent manner, with some studies suggesting a role for chaperones GroEL [7], or SRP [8] to maintain it in an export competent state. Furthermore, β-lactamase is exported post-translationally, [9], whereas MBP is exported when 80% translated [10]. β-Lactamase confers resistance to β-lactam antibiotics and is only functional when properly folded in the periplasm, a property that is useful for measuring export and has been exploited in the previous studies [11,12].

Materials and methods

Cloning of bla into pGBS19 and expression analysis. All work was carried out in the *E. coli* strain DH5α (F-(80dlacZ M15) (lacZYA-argF) U169 hsdR17 (r−m+) recA1 endA1 relA1 deoR). A TEM-1 β-lactamase gene (*bla*), was amplified from the expression vector pMALp2e (New England Biolabs) using primers bla-rev and bla-wt (Table 1), which

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doi:10.1016/j.bbrc.2007.11.093

Table 1
Primers used in this study

Primer	Sequence 5′–3′
Cloning primers	
Bla-wt	CAGTGAATTCTATTGAAAAAGGAAGAGTATG
Bla-opt(B)	ATGAGTATTCAACATTTCCGTGTCGCCCTGATCCCGTTTTTTGC GGCATT TTGCCTGCCGGTTTTTGCT
Bla-opt(I)	CAGTGAATTCTATTGAAAAAGGAAGAGTATGAGTATCCAGCATTTC CGTGTTGCGCTGATCCCGTTCTTCGCGGCATTCTGCCTGCCGGTTTTCGCT
Bla-A1	CAGTGAATTCTATTGAAAAAGGAAGAGTATGAGTATCCAACATTTCCGTGTT GCCCTGATTCCGTTTTTCGCGGCATTTTGCCTGCCTGTTTTCGCTCACCCAG
Bla-A2	CAGTGAATTCTATTGAAAAAGGAAGAGTATGAGTATTCAGCATTTCCGTGT CGCGCTTATCCCCTTCTTTGCGGCATTCTGCCTTCCGGTTTTTGCT
Bla-F6	CAGTGAATTCTATTGAAAAAGGAAGAGTATGAGTATCCAGCATTTCCGTG TTGCGCTGATCCCCTTT
Bla-L7	CAGTGAATTCTATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGT GTCGCCCTTATTCCGTTCTTCGCGGCATTCTGCCTGCCGGTTTTCGCTCACCCAG
F6-deg[a]	CAGTGAATTCTATTGAAAAAGGAAGAGTATGAGTATHCARCATTTCCG TGTNGCNCTNATHCCCTTT
Bla-rev	TTGGCTGCAGATCAACCGGGGTAAATCAAT
RT-PCR primers	
Bla-RTF	TGCCATAACCATGAGTGATA
Bla-RTR	ATCAAGGCGAGTTACATGAT
16S_F	ACGGAGGGTGCGAGCGTTAATC
16S_R	CTGCCTTCGCCTTCGGTATTCCT

[a] Redundant base code: H—ACT, R—AG, N—ACGT.

incorporated EcoRI and PstI sites, respectively. The PCR product was digested and cloned into the multi-cloning site of the vector pGBS19 (based on pUC19, but with a kan^R marker instead of bla; [13]). The cloned *bla* gene included a 24 bp sequence upstream of the start codon containing a Shine Dalgarno sequence, and 79 bp downstream of the stop codon. All subsequent β-lactamase signal sequence mutants were cloned using the same strategy (see Fig. 1A), with the codon changes incorporated into a PCR product using a series of mutagenic primers (Table 1) paired with primer bla-rev.

Expression analysis of β-lactamase. Expression of β-lactamase from these constructs was measured by minimum inhibitory concentration (MIC) to ampicillin of 10^6 cells as described in [14]. The MIC was recorded as the lowest concentration of antibiotic in which no growth was observed after 16 h at 37 °C. The same protocol was used to determine MIC to kanamycin, which, in conjunction with isolation of plasmid DNA from minipreps, was used to monitor plasmid copy number. Plasmid copy number was found to be equivalent for every plasmid construct used in this study. From the same culture used for MIC and plasmid copy number minipreps, a whole cell lysate was made from 10^8 cells and loaded on a 4–12% gradient PAGE gel, and then transferred onto a nitrocellulose membrane. β-lactamase was detected using an 1:10,000 dilution of a anti-β-lactamase polyclonal antibody AB3738 (Chemicon International), and detected using a 1:10,000 dilution of anti-Rabbit IgG (Sigma, A3687) conjugated with alkaline phosphatase. From the same culture, RNA was extracted using a RNeasy miniprep kit (Qiagen) and converted to cDNA using Taqman RTPCR kit. The relative RNA levels to the 16S control were determined by Quantitative RT-PCR on an ABI7700 (Applied Biosystems) using primers Bla-RTF and Bla-RTR to amplify *bla* and 16S_F and 16S_R for the 16S rRNA control.

Selection for β-lactamase genes with higher ampicillin resistance. A degenerate primer called F6_deg was designed (see Fig. 3A) that had degenerate bases at the third position of the first six non-optimal codons of the wild-type *bla* gene. PCR products generated by F6_deg, F6 and Bla-wt primers paired with Bla-rev were digested with EcoRI and PstI and cloned into pGBS19. Prospective colonies were selected on LB-kanamycin (50 μg/ml). Colonies from the F6_deg transformation with higher ampicillin resistance compared to F6 when plated on LB-ampicillin (200 μg/ml) were selected for a second round of cloning experiments. To confirm that only changes within the signal sequence caused this increase in ampicillin resistance, a 330 bp ScaI/EcoRI fragment, from plasmids conferring an increased resistance phenotype, was cloned into pBla-1, a construct that contained a frame-shift mutation in the signal sequence (Fig. 3B). If the introduction of the 330 bp fragment, replacing the frame-shifted signal sequence, conferred increased ampicillin resistance, then the original increased resistance phenotype was due to changes only in the 330 bp of cloned DNA, which was sequenced to confirm the genotype.

The effect of temperature on ampicillin resistance. Cultures of *bla*-wt and *bla*-opt(Ike) were grown at 28 °C in LB-kanamycin until mid-log phase. Cultures were normalized to 1×10^8 cells/ml then 10-fold serially diluted in LB broth and 5 μl of each dilution added to LB-ampicillin (50 μg/ml) plates, or LB-kanamycin (50 μg/ml) for plasmid copy number control, which were incubated at either 28 °C or 37 °C.

Results and discussion

Effect on expression of β-lactamase substituting distinct sets of codons in the signal sequence

Non-optimal codons defined by Burns and Beacham [15] that were present in the *bla* signal sequence, a TEM-1 β-lactamase, were changed to the most optimal codon within the synonymous codon family (Fig. 1B). Expression of β-lactamase from this optimised codon usage allele, *bla*-opt(B), gave a 2-fold higher MIC value, and a corresponding slight increase of β-lactamase on a Western blot (Fig. 1B). This was different to our previous findings with MBP, where an approximate 20-fold reduction of expression in the optimised signal sequence allele was observed [16]. Analysis of the distribution of these non-optimal codons in the β-lactamase signal sequence was done as described previously [4] and showed that most of the non-optimal codons were clustered towards the carboxy-

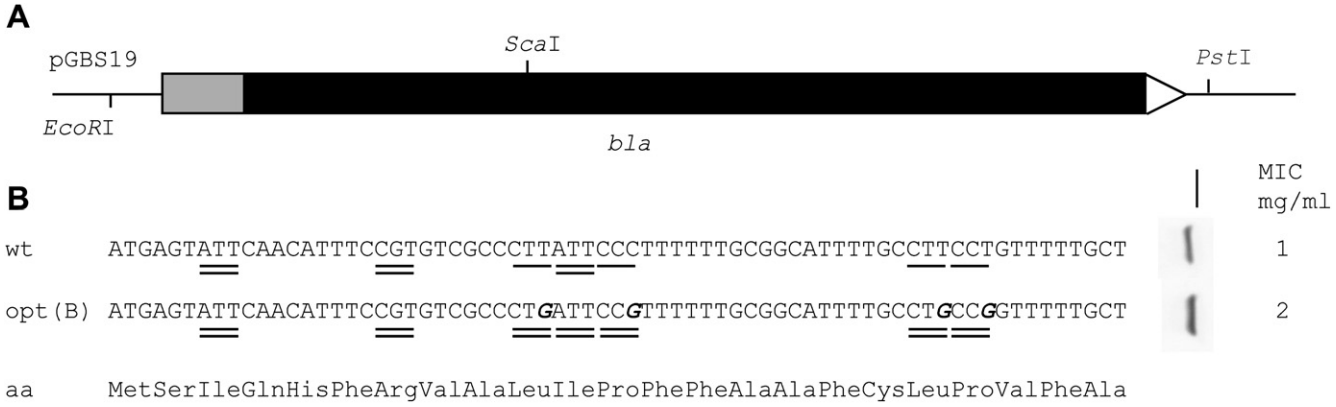

Fig. 1. Phenotype of *bla* expression based on Burns and Beacham non-optimal codons. (A) Schematic of *bla* gene with cloning sites used in this study indicated on the gene. The grey box indicates the signal sequence while the black box shows the mature *bla* gene. (B) The optimised β-lactamase signal sequence replacing Burns and Beacham [15] non-optimal codons. The Western blot phenotype of each strain is shown to the right of the sequence, along with corresponding MIC values. MIC for kanamycin was 8 mg/ml for all strains, confirming equivalent plasmid copy number. The vertical black line indicates molecular weight marker at 37.1 kDa (Invitrogen, Cat. No. 10748-010). Non-optimal and optimal codons are indicated by single and double underline, respectively. Letters in bold and italic indicate nucleotide changes from the wild-type sequence.

terminal end of the signal sequence, adjacent to the cleavage site (Fig. 2A). This distribution is the opposite of the genomic mean of all secretory genes in *E. coli*, where non-optimal codons are most abundant in the amino-terminal end of the signal sequence [4].

The definitions of *E. coli* non-optimal codons vary from study to study depending on the criteria used [15,17–20]. In general, non-optimal codons are defined by slower rate of translation either due to low cognate tRNA concentrations or inefficient codon–anticodon interactions. The non-optimal codons used by Burns and Beacham [15] were defined by frequency of codon use and intracellular tRNA concentrations, which described non-optimal codons in seven amino acid families. Ikemura [20] described a larger set of non-optimal codons defined by the efficiency of the codon–anticodon interactions in addition to tRNA concentrations in fourteen amino acid families. Inefficient codon–anticodon interactions have been shown to cause translational pausing [21,22]. Sliding window analysis of the distribution of larger Ikemura [20] non-optimal codon set in the signal sequence of β-lactamase revealed >50% non-optimal codons in the N-terminal half of the signal sequence. This is more consistent with the average genomic distribution of non-optimal codons in *E. coli* secretory genes, which is above 50% in the 5′-end of the signal sequence [4] and trails down to the genomic mean for all genes of ~30% at the 3′-end (Fig. 2). When the 13 non-optimal Ikemura codons found in the *bla* signal sequence were changed to an optimal codon from the same synonymous codon family, expression of β-lactamase from this allele, *bla*-opt(I), gave a 4-fold reduction in MIC compared to *bla*-wt. The drop in expression was not due to changes in plasmid copy number, or *bla* mRNA levels, which were found to be equivalent by quantitative real time PCR (data not shown). This data confirmed that non-optimal codons were important for expression of *bla*, and also suggested that the Ikemura non-optimal codon set [20] were functionally equivalent to the Burns and Beacham set [15] used in our previous MBP study [4].

To determine whether the distribution of the non-optimal codons within the signal sequence had functional significance in expression of β-lactamase, four signal sequence mutants were made in which either the first six or last seven non-optimal codons (*bla*-F6, *bla*-L7), or every alternate non-optimal codon (*bla*-A1, *bla*-A2) were changed to optimal (Fig. 2). Analysis of expression by Western blot and MIC revealed the following rank order for expression (lowest to highest) of the *bla* alleles used in this study: *bla*-opt(I) < *bla*-F6, *bla*-A2 < *bla*-A1, *bla*-wt < *bla*-L7, *bla*-opt(B).

These results confirm that the distribution of non-optimal codons within the signal sequence is important to maintain expression of *bla*. The drop in expression of the *bla*-F6 allele compared to *bla*-wt confirms that non-optimal codons in the N-terminal half of the signal sequence are important to maintain expression of *bla*. The increase in expression of the *bla*-L7 allele compared to *bla*-wt might also be explained through non-optimal codon distribution. This mutant, which still contains the N-terminal half non-optimal codons, more closely matches the drop in non-optimal codons observed in the average genomic distribution at the 3′-end of the signal sequence [4], which is not present in the *bla*-wt allele (Fig. 2A). Combined, these results confirm that non-optimal codons in the N-terminal half more are important in maintaining expression of *bla* than C-terminal ones.

Selection for revertants with higher MIC values

To test the hypothesis that non-optimal codons in the N-terminal half of the β-lactamase signal sequence are key in maintaining optimal β-lactamase expression, a degenerate primer F6_deg was designed that had redundancies at the third position of the first six non-optimal

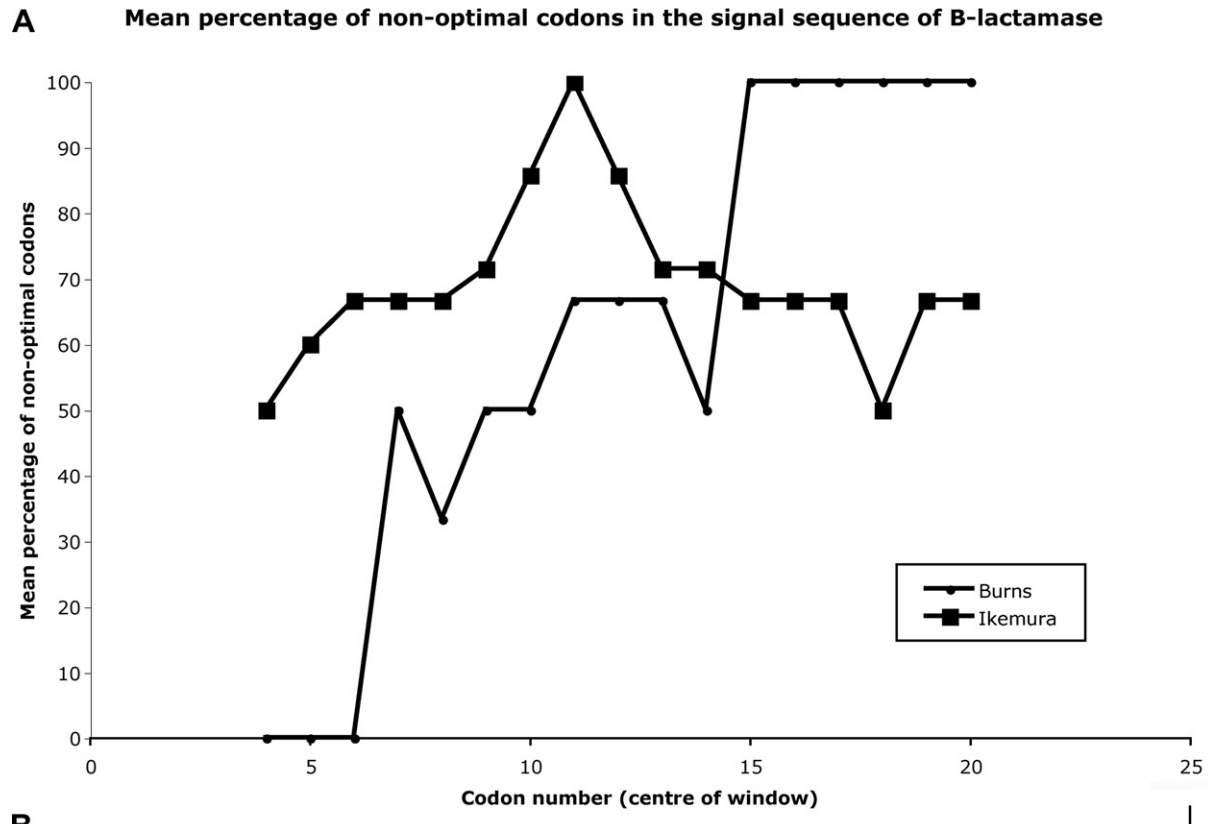

Fig. 2. Phenotype of *bla* expression based on Ikemura non-optimal codons. (A) Plot indicating the mean percentage of non-optimal codons in the signal sequence of β-lactamase, according to Ikemura [20] and Burn and Beacham [15] non-optimal codon sets calculated using a seven-codon sliding window. The data points are reported in the middle of the sliding window (position 4) and are calculated using the formulae: no. non-optimal/(no. non-optimal + no. optimal). (B) Changes to the β-lactamase signal sequence using Ikemura non-optimal codon set. Letters in bold and italic indicate nucleotide changes from the wt sequence. The Western blot phenotype of each mutant is shown to the right of the sequence, followed by the corresponding MIC value. MIC for kanamycin was 8 mg/ml for all strains, confirming equivalent plasmid copy number. The vertical black line indicates the 37.1 kDa molecular weight marker (Invitrogen, Cat. No. 10748-010). Non-optimal and optimal codons are indicated by single and double underline, respectively.

codons in the wild-type *bla* gene (Fig. 3). This allows selection of revertants that shift between non-optimal and optimal codons, but does not change the amino acid sequence. The codons TTC and CGT were not changed, as they are already optimal in the wild-type *bla* gene. Using CTN to cover the leucine family, a complexity of 1152 possible codon combinations was possible. Using a two-round cloning strategy (see Materials and methods), nine colonies with increased ampicillin resistance were identified and sequenced (Fig. 3). All nine colonies contained reversions to non-optimal codons; six clones contained reversions to non-optimal codons as defined by Ikemura [20] at four positions in the sequence, two clones had two non-optimal codon reversions, and one had a single non-optimal codon

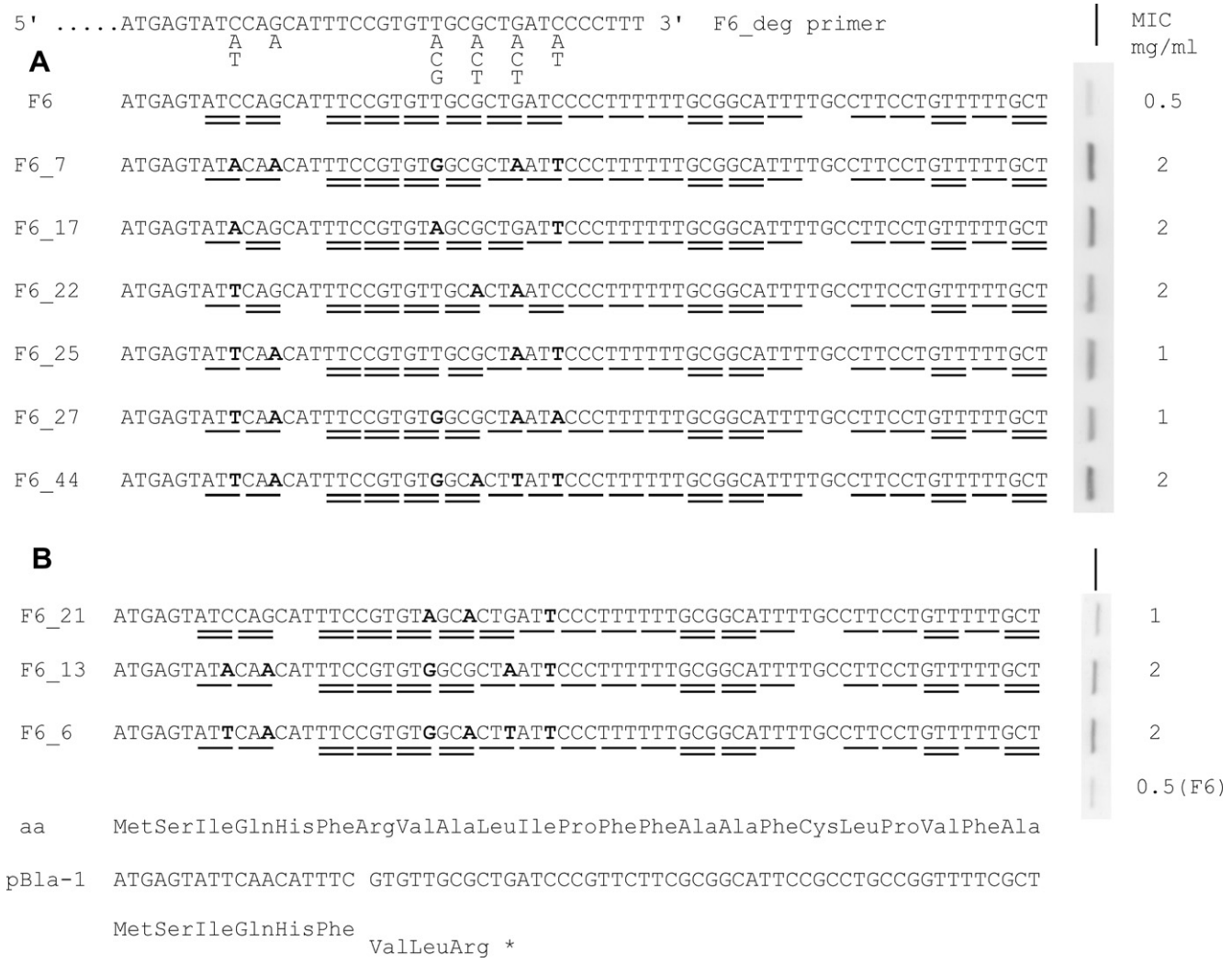

Fig. 3. Analysis of revertant signal sequences with increased ampicillin resistance. Sequence of the mutagenic F6_deg primer, indicating the full range of possible codon combinations, is shown at the top of the figure. (A) and (B) show revertant sequences identified from two separate cloning experiments. Nucleotide sequence changes of the revertant colonies that differ to *bla*-F6 sequence are shown in bold. Non-optimal and optimal codons as defined by Ikemura [20] are indicated by single and double underline, respectively. The Western blot phenotype of each mutant is shown to the right of the figure, with the corresponding MIC value next to it. MIC for kanamycin was 8 mg/ml for all strains, confirming equivalent plasmid copy number. The vertical black line indicates the 37.1 kDa molecular weight marker (Invitrogen, Cat. No. 10748-010). Underneath is the sequence of the frame-shifted *bla* gene in plasmid pBla-1, with the frame-shifted amino acid sequence until the first stop codon appears.

reversion. Some of the non-optimal codon reversions were different to the codons found in the original wild-type *bla* gene. Eight of the nine revertant colonies contained the non-optimal codons ATA or ATT for isoleucine at codon 3, and six had the non-optimal CAA codon for glutamine at codon 4. The single reversion clone, F6_21, with an ATT at codon 11 had the weakest *bla* expression of all the colonies isolated. In comparison, the F6_17 clone, with the same reversion at codon 11, and an ATA at codon 3, has much stronger expression. The results of this reversion study strongly suggest that non-optimal codons at the amino terminal half of the signal peptide are more important for efficient expression of secretory proteins. This is supported by a similar finding in MBP, which had an increase in expression observed in an MBP-opt construct when the optimal ATC at codon 3 changed the non-optimal ATA (unpublished data).

Growth of bla-opt(Ike) enhanced at 28 °C compared to 37 °C

Previous studies have shown that the degradation of abnormal proteins can occur at a lower rate at 30 °C than at 37 °C [23,24]. Growth at lower temperatures has also been used to facilitate the production of recombinant proteins in *E. coli* [25]. The reduction in temperature has the effect of reducing the activity of heat-shock regulated proteases [26]. Our previous study demonstrated that expression of the optimised MBP (MBP-opt) could be recovered by expressing in heat-shock protease deficient strains [6]. The

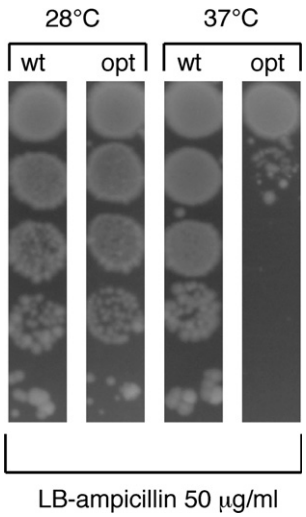

Fig. 4. Temperature effect on growth of bla-opt(Ike) on LB-ampicillin plates. Cultures of *bla*-wt and *bla*-opt(Ike) were grown at 28 °C in LB-kanamycin and normalised to 1×10^7 cells/ml. These were 10-fold serially diluted in LB and a 5 μl drop added LB-ampicillin plates (50 μg/ml) and incubated at either 28 or 37 °C.

expression of MBP-opt was recovered completely when grown at 28 °C (Zalucki & Jennings, unpublished observations) indicating that expression at this lower temperature was equivalent to expression in a multiple protease deficient strain [6]. To see if expression of *bla*-opt(Ike) was similarly temperature dependent, the MIC was done at 28 °C which showed that there was only a 2-fold difference between compared to the 4-fold difference seen at 37 °C (data not shown). As the MIC test can only resolve to a 2-fold difference in ampicillin resistance, to achieve more sensitivity a plating efficiency assay over a range of inocula was performed (Materials and methods). These results showed that *bla*-opt(Ike) was dramatically different at 37 °C but had a wt phenotype at 28 °C at the concentration of ampicillin used (50 μg/ml) (Fig. 4), indicating both expression and export of the optimised *bla* allele is improved at 28 °C. This suggests that the difference in expression between *bla*-opt(Ike) and *bla*-wt observed at 37 °C is post-translational, due to degradation of a misfolded conformation of Bla-opt(Ike). The increase in expression and export of *bla*-opt(Ike) at 28 °C, coupled with the general observation that abnormal proteins can be stable at lower temperatures (Zalucki & Jennings, unpublished observations, [23,24]) supports this conclusion.

An alternative hypothesis is that the altered codon usage has the potential to promote mRNA secondary structure that may alter gene expression. These structures could be formed due to increase in the G/C content in the signal sequence as a consequence of non-optimal to optimal codon changes [27]. Should such structures exist they would be more stable at 28 °C than at 37 °C, and would result in less or equivalent expression of *bla*-opt(Ike) at this lower temperature. The observation that *bla*-opt(Ike) grows better on LB-ampicillin plate at 28 °C than 37 °C suggests that inhibitory RNA secondary structures are not formed, and are not responsible for the reduction in *bla* expression. Also the revertant study revealed that non-optimal codons, some different to wild-type *bla* gene, gave increased β-lactamase expression. It is unlikely that these combinations of non-optimal codons are all involved in favourable mRNA secondary structures.

This study, along with the previous one in MBP [6], has demonstrated that non-optimal codons have a key role in maintaining optimal expression of two secretory proteins. Given the two different export pathways of MBP and β-lactamase, the translational pausing due to non-optimal codons could be important for SecB-dependent and SecB-independent modes of export.

Acknowledgments

Thanks to Prof. Ifor Beacham for his comments on this manuscript. M.P.J. lab is supported by NHMRC program Grant 284214.

References

[1] T. Ikemura, Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes, J. Mol. Biol. 146 (1981) 1–21.
[2] T.A. Thanaraj, P. Argos, Protein secondary structural types are differentially coded on messenger RNA, Protein Sci. 5 (1996) 1973–1983.
[3] A.A. Komar, T. Lesnik, C. Reiss, Synonymous codon substitutions affect ribosome traffic and protein folding during in vitro translation, FEBS Lett. 462 (1999) 387–391.
[4] P.M. Power, R.A. Jones, I.R. Beacham, C. Bucholtz, M.P. Jennings, Whole genome analysis reveals a high incidence of non-optimal codons in secretory signal sequences of *Escherichia coli*, Biochem. Biophys. Res. Commun. 322 (2004) 1038–1044.
[5] Y.D. Li, Y.Q. Li, J.S. Chen, H.J. Dong, W.J. Guan, H. Zhou, Whole genome analysis of non-optimal codon usage in secretory signal sequences of *Streptomyces coelicolor*, Biosystems 85 (2006) 225–230.
[6] Y.M. Zalucki, M.P. Jennings, Experimental confirmation of a key role for non-optimal codons in protein export, Biochem. Biophys. Res. Commun. 355 (2007) 143–148.
[7] E.S. Bochkareva, N.M. Lissin, A.S. Girshovich, Transient association of newly synthesized unfolded proteins with the heat-shock GroEL protein, Nature 336 (1988) 254–257.
[8] G.J. Phillips, T.J. Silhavy, The *E. coli* ffh gene is necessary for viability and efficient protein export, Nature 359 (1992) 744–746.
[9] D. Koshland, D. Botstein, Evidence for posttranslational translocation of beta-lactamase across the bacterial inner membrane, Cell 30 (1982) 893–902.
[10] L.L. Randall, Translocation of domains of nascent periplasmic proteins across the cytoplasmic membrane is independent of elongation, Cell 33 (1983) 231–240.
[11] J.T. Kadonaga, A. Pluckthun, J.R. Knowles, Signal sequence mutants of beta-lactamase, J. Biol. Chem. 260 (1985) 16192–16199.
[12] A. Pluckthun, I. Pfitzinger, Membrane-bound beta-lactamase forms in *Escherichia coli*, J. Biol. Chem. 263 (1988) 14315–14322.
[13] B.G. Spratt, P.J. Hedge, S. te Heesen, A. Edelman, J.K. Broome-Smith, Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9, Gene 41 (1986) 337–342.
[14] Y.M. Zalucki, P.M. Power, M.P. Jennings, Selection for efficient translation initiation biases codon usage at second amino acid position in secretory proteins, Nucleic Acids Res. 35 (2007) 5748–5754.
[15] D.M. Burns, I.R. Beacham, Rare codons in *E. coli* and S. typhimurium signal sequences, FEBS Lett. 189 (1985) 318–324.

[16] Y.M. Zalucki, M.P. Jennings, Experimental confirmation of a key role for non-optimal codons in protein export, Biochem. Biophys. Res. Commun. (2007).
[17] S. Pedersen, *Escherichia coli* ribosomes translate in vivo with variable rate, EMBO J. 3 (1984) 2895–2898.
[18] P.M. Sharp, W.H. Li, Codon usage in regulatory genes in *Escherichia coli* does not reflect selection for 'rare' codons, Nucleic Acids Res. 14 (1986) 7737–7749.
[19] S.P. Zhang, G. Zubay, E. Goldman, Low-usage codons in *Escherichia coli*, yeast, fruit fly and primates, Gene 105 (1991) 61–72.
[20] T. Ikemura, Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes: a proposal for a synonymous codon choice that is optimal for the *E. coli* translational system, J. Mol. Biol. 151 (1981) 389–409.
[21] L.K. Thomas, D.B. Dix, R.C. Thompson, Codon choice and gene expression: synonymous codons differ in their ability to direct aminoacylated-transfer RNA binding to ribosomes in vitro, Proc. Natl. Acad. Sci. USA 85 (1988) 4242–4246.
[22] J.F. Curran, M. Yarus, Rates of aminoacyl-tRNA selection at 29 sense codons in vivo, J. Mol. Biol. 209 (1989) 65–77.
[23] M.J. Kosinski, U. Rinas, J.E. Bailey, Proteolytic response to the expression of an abnormal beta-galactosidase in *Escherichia coli*, Appl. Microbiol. Biotechnol. 37 (1992) 335–341.
[24] M.J. Kosinski, J.E. Bailey, Temperature and induction effects on the degradation rate of an abnormal beta-galactosidase in *Escherichia coli*, J. Biotechnol. 18 (1991) 55–68.
[25] J.A. Chesshyre, A.R. Hipkiss, Low-temperatures stabilize interferon-alpha-2 against proteolysis in methylophilus–methylotrophus and *Escherichia coli*, Appl. Microbiol. Biotechnol. 31 (1989) 158–162.
[26] D.B. Straus, W.A. Walter, C.A. Gross, *Escherichia coli* heat shock gene mutants are defective in proteolysis, Genes Dev. 2 (1988) 1851–1858.
[27] D.P. Humphreys, M. Sehdev, A.P. Chapman, R. Ganesh, B.J. Smith, L.M. King, D.J. Glover, D.G. Reeks, P.E. Stephens, High-level periplasmic expression in *Escherichia coli* using a eukaryotic signal peptide: importance of codon usage at the 5′ end of the coding sequence, Protein Expr. Purif. 20 (2000) 252–264.