1    **Synonymous lysine codon usage modification in a mobile antibiotic resistance gene**

2    **similarly alters protein production in bacterial species with divergent lysine codon usage**

3    **biases because it removes a duplicate AAA lysine codon.**

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17    **Abstract**

18    **The mobile antibiotic resistance gene $bla_{IMP-1}$ is clinically important and has a synonymous**

19    **AAA:AAG lysine codon usage bias of 73:27. This bias is like that seen in experimentally**

20    **determined highly expressed genes in *Escherichia coli* and *Acinetobacter baumanii*, but**

21    **quite different from that seen in *Pseudomonas aeruginosa* (26:74 AAA:AAG). Here we**

22    **show that, paradoxically, shifting the AAA:AAG lysine codon bias to 8:92 in $bla_{IMP-1}$**

23    **expressed from a natural promoter results in significantly more IMP-1 production in all**

24    **three species.  Sequential site directed mutagenesis revealed that increased IMP-1**

25    **production occurs following removal of an AAA,AAA double lysine codon and that**

26    **otherwise, lysine codon usage had no observable impact on IMP-1 production. We**

27    **conclude that ribosomal slippage at this poly-adenosine region reduces efficient**

28    **translation of IMP-1 and that punctuating the region with guanine reduces ribosomal**

29    **slippage and increases IMP-1 production.**

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## Introduction

Synonymous codon usage bias (SCUB) is a term describing the common finding that

organisms favour the use of certain triplet codons in DNA to encode certain amino acids.

Since SCUB varies between organisms, and between different genes in a single organism,

the implication is that optimal SCUB varies between different organisms, and that certain

genes are selected to be closer to the optimal SCUB than others (1). One dominant

hypothesis is that highly expressed genes have "optimised" SCUB and that this is selected

because optimal codons are translated more quickly and/or more efficiently than sub-

optimal ones. This is particularly important when the demand for a protein is high (1).

Indeed, it is well known that SCUB optimization – adapting the SCUB of a recombinant gene

to match that of highly expressed genes – increases recombinant protein production in a

heterologous host (2). The success of this methodological approach has been used to

advance the translationally-selective hypothesis to explain SCUB. However, most codon

usage optimization procedures involve the over-expression of recombinant genes using

hyper-strong, inducible promoters and high copy number vectors, with a desire to make a

single protein represent a high percentage of total protein in the cell (3). This is not likely to

reflect the situation encountered by a gene in a natural setting with a natural promoter on

the chromosome or a low copy number plasmid.

SCUB is particularly relevant in the context of horizontal gene transfer. Whilst sub-optimal

SCUB is not always seen in horizontally acquired genes, depending on their origins, for those

that are sub-optimal, selective pressure is expected to be applied over time to optimise

SCUB; a process referred to as "codon usage amelioration" (4). Antibiotic resistance in

bacteria is one of the most pressing threats to human health and horizontal gene transfer is

55   one of the most important means for a bacterium to acquire antibiotic resistance (5). It is

56   evident that many mobile genetic elements are of low guanine plus cytosine (GC) content

57   and carry a related SCUB biased towards low GC codons (6). So, in this context, the impact

58   of SCUB on gene expression is not only academic, but it is also of significant practical

59   interest. The aim of the work reported in this paper was to test the hypothesis that SCUB

60   change can affect the absolute amount of active protein produced in a predictable manner

61   using a natural plasmid system and an intermediate strength natural promoter using a

62   clinically important, mobile antibiotic resistance gene.

63

## Results and Discussion

65   The $bla_{IMP-1}$ metallo-β-lactamase-encoding gene cassette, which confers resistance to the

66   carbapenems, a class of "last resort" antibiotics, has a GC content of 39% (7). However, it

67   has been found widely among Gram-negative bacteria with varying genomic GC contents

68   (8).  For example, it is commonly found in *Escherichia coli* and other Enterobacteriaceae

69   which have genomic GCs of ≈50%. Accordingly, if SCUB affects translational efficiency

70   and/or rate, one would expect the amount if IMP-1 enzyme should be seen to increase in

71   Enterobacteriaceae if the SCUB of $bla_{IMP-1}$ is ameliorated to match the optimal SCUB of this

72   family, as represented by *E. coli*. To test this, specific imipenemase activity was measured in

73   extracts of *E. coli* MG1655 transformants carrying pHIMP or pH*Ec*IMP, being the cloned

74   wild-type or *E. coli* SCUB optimised $bla_{IMP-1}$ gene cassette, respectively, each under the

75   control of an identical, natural, intermediate-strength integron promoter, and each ligated

76   into a broad host-range, low copy number vector derived from a natural antibiotic

77   resistance plasmid: RK2 (9). We can confirm that pHIMP is a truly natural expression system

78  because imipenemase activity in an MG1655 transformant carrying pNIMP (the natural

79  *bla*$_{IMP-1}$ encoding plasmid) and MG1655(pHIMP) were the same (**Figure 1**). If the hypothesis

80  being tested is correct, it was expected that optimisation of *bla*$_{IMP-1}$ to a SCUB closer to

81  optimal in *E. coli* genes would increase IMP-1 production relative to wild-type. However, this

82  was not observed. The pH*Ec*IMP variant synthesised to match the "optimal" SCUB of *E. coli*

83  presumed highly expressed genes (ribosomal protein and translation elongation factor

84  genes) based on the OPTIMIZER algorithm (10) was expressed at lower levels than than the

85  wild-type gene (p<0.0001) (**Figure 1**).

86  It was considered possible that the effect seen here is due to stability changes at the 5' end

87  of the codon-optimised *bla*$_{IMP-1}$ mRNA. Strong regions of secondary structure at the 5' ends

88  of mRNA molecules are likely to cause ribosomal occlusion leading to the exposure of mRNA

89  to nuclease digestion (11). Furthermore, it has previously been shown that synonymous

90  mutations that increase mRNA secondary structure (high folding energy) at the beginning of

91  the transcript can reduce protein production by inhibiting the initiation and initial phase of

92  translation elongation (12-14). It was confirmed that codon optimization increased the

93  energy required to unfold the mRNA. The Gibbs free energy value of the whole mRNA

94  molecule was -183 (wild type *bla*$_{IMP-1}$) changing to -229 for the *E. coli* codon optimised

95  variant. Just looking at the 5' third of the mRNA, which is thought to be particularly

96  important, the folding energy calculated showed the same effect: moving from -50 for wild-

97  type *bla*$_{IMP-1}$ to -67 for the *E. coli* variant.

98  **Figure 1** shows evidence, therefore, that codon optimization can have negative effects on

99  gene expression in a natural expression system, but it was considered of interest to see how

100  much of a change in IMP-1 production would occur upon site-directed mutation of

101    individual codons. Twenty six of 246 (10.6%) of IMP-1's amino acids are lysine. The lysine

102    codon AAA is in the majority in $bla_{IMP-1}$ (19/26; 73%) and AAG accounts for the rest. Rather

103    than relying on theoretical lists of "highly expressed genes" to define the "optimal" SCUB for

104    lysine codons in $bla_{IMP-1}$, we measured protein abundance using LC-MS/MS proteomics. In so

105    doing we defined the 20 most highly abundant proteins in three test species during growth

106    under the conditions we would also use to test IMP-1 production (**Tables 1-3**).  Analysis of

107    lysine SCUB in the genes encoding these 20 proteins from each species revealed an AAA

108    percentage of 84% for *Acinetobacter baumannii*, 80% for *E. coli* and 24% for *Pseudomonas*

109    *aeruginosa* (**Tables 4-6**). We sub-cloned the wild-type $bla_{IMP-1}$ gene from pHIMP into the

110    pSU18 cloning vector and used site directed mutagenesis to dramatically reduced the AAA

111    lysine codon usage of $bla_{IMP-1}$ in the resultant pSUHIMP-WT plasmid to 2/26 or 8% AAA in

112    plasmid pSUHIMP-KV. Plasmids were used to transform an *E. coli* clinical isolate to

113    chloramphenicol resistance. The wild-type and lysine codon-variant $bla_{IMP-1}$ genes were also

114    sub-cloned into the broad host-range vector pUBYT, generating pUBYTHIMP-WT and

115    pUBYTHIMP-KV, which were used to transform *P. aeruginosa* PA01 or *A. baumannii* CIP 70-

116    10 to kanamycin resistance. We then used proteomics to measure the abundance of IMP-1

117    in these transformants, which was normalised using the abundance of vector-encoded Cat

118    (in *E. coli* pSUHIMP-WT and -KV transformants) or AphA (in *A. baumannii* and *P. aeruginosa*

119    pUBYTHIMP-WT and -KV transformants) to take into consideration plasmid copy number

120    and protein loading. Our expectation given the hypothesis that SCUB is selected based on

121    translation rate or efficiency was that as AAA usage was reduced to 2/26 from a wild-type

122    position of 19/26, which is close to optimal in *A. baumannii* (22/26) and *E. coli* (21/26) there

123    would be a reduction in IMP-1 production. The case in *P. aeruginosa* was not so clear, given

124    that the optimal AAA usage in this species is 6/26. Here, the variant is closer to optimal than

125    the wild-type gene, so we might expect an increase in IMP-1 production. We did see this: a

126    1.5-fold increased normalised IMP-1 production in PA01(pUBYTHIMP-KV) compared with

127    PA01(pUBYTHIMP-WT), $p$=0.04 for an unpaired t-test, $n$=3. However, the variant also

128    supported higher IMP-1 protein production in *E. coli* (2.2-fold, $p$=0.005 $n$=3) and *A.*

129    *baumannii* (3.2-fold, $p$=0.002, $n$=3) (**Figure 2**).

130    These data show that the simple idea of optimization based on average SCUB – even when

131    that average is taken from highly expressed genes confirmed by proteomics – is rather

132    naïve. The codons in an mRNA affect its folding, which affects its stability and the rate of

133    translation initiation and elongation; local and global charged tRNA levels affect translation

134    elongation rate, and this must be optimised and even varied during the translation of an

135    mRNA to allow accurate protein folding (11-14). Our finding of increased IMP-1 production

136    when AAG lysine codons dominate is not due to relative tRNA abundance since there is only

137    one lysine-tRNA, which recognises both AAA and AAG codons (15). Lysine tRNA/codon

138    specific nucleases have been reported in *E. coli* (16), and it is conceivable that AAA/tRNA

139    interactions preferably promote mRNA cleavage, but the effect we report was seen in three

140    very distinct species, and there is no evidence that AAG/anticodon interactions mean less

141    cleavage, even in *E. coli* (16). The most likely explanation for our findings is a report that

142    duplicate AAA lysine codons lead to ribosomal sliding and increased aberrant translation of

143    an mRNA in *E. coli* (17). We analysed the concatenated sequence data for the genes

144    encoding the 20 most abundant proteins in *E. coli* and found that there are eleven AAA,AAA

145    double lysine codons, comprising 7.8% of all AAA codons. In contrast there are eleven

146    AAA,AAG or AAG,AAA and two AAG,AAG double lysine codons and one AAG,AAG,AAG triple

147    lysine codon found amongst these 20 genes, comprising 26.1% of all AAG codons. This

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148    suggests that there is selective pressure for the inclusion of AAG codons preferentially

149    where two or more lysines are encoded together. Within the 17 AAA to AAG mutations

150    made in our lysine codon modified $bla_{IMP-1}$ gene (**Figure 3**) one is part of an AAA,AAA double

151    lysine codon, with both codons being converted into AAG in the same mutagenesis step

152    (mutations 6 and 7). To test the specific effects of this mutagenesis step, we measured

153    imipenemase activity in cell extracts of *E. coli* MG1655 transformants carrying pSUHIMP

154    variants having an accumulating number of AAA to AAG mutations, from 1 to 17, starting at

155    the 5' end of the gene. **Figure 4** shows that carrying $bla_{IMP-1}$ with 6 or more mutations gives

156    levels of IMP-1 enzyme activity not significantly different from that provided by pSUHIMP-

157    KV, having all 17 mutations ($p>0.1$). Importantly, introduction of mutations 6 and 7, where

158    IMP-1 enzyme activity significantly increases from basal ($p<0.03$) is the point at which the

159    AAA,AAA double lysine codon is converted to AAG,AAG. Therefore, based on previously

160    published work using in vitro translation experiments (17), we conclude that there is

161    ribosomal slippage at the AAA,AAA run located in the $bla_{IMP-1}$ mRNA, reducing the amount

162    of active IMP-1 protein produced. Breaking up this run with AAG codons means more

163    correct translation and so more IMP-1 enzyme activity. Importantly, we see this effect in all

164    three species tested, despite their divergence. There is no evidence for mutation in the

165    AAA,AAA run in any $bla_{IMP-1}$ variant sequence in the Genbank nucleotide sequence database,

166    according to blastn, so the increased IMP-1 enzyme production stimulated by this mutation

167    is seemingly not under strong selective pressure *in vivo*. However, there are two IMP

168    variants where the second lysine codon in this run has been mutated in a non-synonymous

169    way. The most common of these is IMP-22 (18).

8

170    In conclusion, codon "optimisation" and mutations that change SCUB to be more closely

171    aligned or more distantly aligned to highly expressed genes in multiple bacteria do not

172    guarantee higher levels of gene expression. Indeed, for synonymous lysine codon changes,

173    the increase in IMP β-lactamase production when SCUB was moved further from "optimal"

174    is paradoxical and is most likely to be cause by reduced aberrant protein production that

175    occurs when AAA codons are present in duplicate (17). Care should therefore be taken when

176    interpreting the potential impact of synonymous mutations that affect codon usage in

177    horizontally acquired genes carried on natural plasmids and expressed from native

178    promoters without experimental determination of the effect of these mutations on protein

179    abundance or some phenotypic proxy thereof.

180

181    **Experimental**

182    Bacterial Strains

183    Bacterial strains used in the study were *E. coli* TOP10 (Invitrogen), MG1655 (19) and a clinical

184    isolate from urine (a gift from Dr Mandy Wooton, Public Health Laboratory for Wales); *P.*

185    *aeruginosa* PA01 (20) and *A. baumanii* CIP 70-10 (21)

186    Molecular Biology

187    The $bla_{IMP-1}$ gene was amplified using PCR. Template DNA was extracted from *P. aeruginosa*

188    clinical isolate 206-3105A (a gift from Dr Mark Toleman, Department of Medical

189    Microbiology, Cardiff University) by suspending a loop-full of bacteria from a fresh Nutrient

190    Agar plate (Oxoid) in 100 μl of molecular biology grade water. The tube was then incubated

191    at 95°C for 15 min and centrifuged at 13,000 rpm for 10 min. The supernatant was removed

192    as a source of template DNA. The integron promoter type upstream of $bla_{IMP-1}$ in isolate

193     206-3105A is PcH1 (22), and there is a $bla_{OXA-2}$ gene cassette downstream from $bla_{IMP-1}$

194     (Genbank accession: AP012280.1). PCR used forward primers which were designed to

195     amplify from the 5' end of the wild-type PcH1 promoter (5'-

196     ACCCAGTGGACATAAGCCTGTTCGGTTCGTAAACT-3') into the 5' end of the $bla_{OXA-2}$ gene

197     cassette, (5'-AGCGAAGTTGATATGTATTGTG-3'). Each PCR reaction mixture contained 20 ng

198     of template DNA, 0.4 μmol of each primer, 12.5 μl of RedTaq PCR-ready reaction mix

199     (Sigma-Aldrich) and 8.5 μl of molecular biology grade water. PCR reactions were processed

200     in PTC-100 thermal cycler (Bio-Rad, UK) in 0.2 ml PCR tubes (Starlabs). PCR reaction cycles

201     were 10 min at 95°C, followed by 35 cycles of, 1 min denaturation at 95°C , 1 min annealing

202     at 58°C and 2 min extension at 72°C. The final step was an extension at 72°C for 10 min. The

203     PCR amplicon was TA cloned into the pCR2.1TOPO cloning vector (Invitrogen), removed with

204     EcoRI and ligated into EcoRI linearised RK2-derived vector pRW50 (9) to create the

205     recombinant plasmid pHIMP or the broad host range p15A derived vector pSU18 (23) to

206     create the recombinant plasmid pSUHIMP.

207     The *E. coli* codon optimized $bla_{IMP-1}$ gene variant was designed using the program

208     OPTIMIZER (10) and the variant, including up- and down-stream sequences identical to

209     those seen in pHIMP was synthesized by GeneArt (Thermo-Fisher) and provided, cloned into

210     the cloning vector pMK as the vector pMKH*Ec*IMP. The optimised gene was amplified by PCR

211     using pMKH*Ec*IMP as template and cloned into pRW50, as described for the wild-type gene

212     to create the recombinant plasmid pH*Ec*IMP.

213     Site directed mutagenesis was performed using the QuikChange® Lightning Site-Directed

214     Mutagenesis Kit (Agilent, UK) according to the manufacturer's instructions and pSUHIMP as

215     the template. The 17 individual AAA to AAG mutations (**Figure 3**) were introduced in 14

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216     separate mutagenesis steps, each creating a variant with an increasing number of mutations

217     starting at the 5' end of the gene. The primers were designed using the mutagenesis kit

218     manufacturer's instructions and are shown in **Table 7**.

219     For transformation of *A. baumannii* and *P. aeruginosa bla*$_{IMP-1}$ wild type and variants were

220     subcloned into vector pUBYT being the plasmid pYMAb2 (24) which we modified to remove

221     the OXA promotor region (located upstream of the multiple cloning site) by PCR

222     amplification using primers, 5'-GCAAGAAGGTGATGAATCTACA-3' and 5'-

223     GTGGCAGCAGCCAACTCA-3' followed by digestion with XbaI and ligation to produce a

224     circular product.

225     <u>Measuring imipenemase specific activity in cell extracts</u>

226     A volume of 0.5 ml of overnight nutrient bacterial broth culture was added to a 10 ml of

227     fresh nutrient broth which was incubated at 37°C with shaking until an OD$_{600}$ of 0.5-0.6 was

228     reached. The cells were then pelleted by centrifugation at 4,500 rpm for 10 min at 4°C. The

229     pellet was re-suspended in 1 ml of 50 mM HEPES (containing 100 µM ZnCl$_2$ at pH 7) and

230     transferred to a tube of lysing matrix B (Fisher Scientific, UK). The cells were lysed using a

231     Ribolyser (Hybaid, UK) at speed of 6.0 for 40 s followed by centrifugation at 13,000 rpm for

232     1 min to pellet cell debris. The supernatant was used for enzyme activity measurement.

233     Total protein concentration was determined using the Bio-Rad protein assay reagent

234     according to the manufacturer's instructions. To measure the imipenemase activity in an

235     extract, 100 µl of extract was added to 900 µl of HEPES buffer (containing ZnCl$_2$, as above)

236     and 0.1 mM imipenem. Change of absorbance was monitored at 299 nm over 10 min.

237     Specific enzyme activity (pmol imipenem hydrolysed per mg of protein per sec) in each

238    extract was calculated using 7000 M$^{-1}$ as the extinction coefficient of imipenem and dividing

239    enzyme activity with the total amount of protein in each assay.

240    mRNA Secondary Structure Prediction

241    To assess the presence of significant secondary structure in the transcript of wild-type

242    $bla_{IMP-1}$ and the *E. coli* SCUB optimized variant, the Mfold program

243    (http://unafold.rna.albany.edu/?q=mfold) was used to predict the folding of the mRNA

244    sequences.

245    Preparation of samples from cultured bacteria and proteomics analysis

246    Bacterial cultures were incubated 50 ml Nutrient Broth (Sigma) with shaking (160 rpm) at

247    37°C until OD$_{600}$ reached 0.6-0.8. Cells in cultures were pelleted by centrifugation (10 min,

248    4,000 × g, 4°C) and resuspended in 35 mL of 30 mM Tris-HCl, pH 8 and broken by sonication

249    using a cycle of 1 sec on, 1 sec off for 3 min at amplitude of 63% using a Sonics Vibracell VC-

250    505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were

251    centrifuged at 8,000 rpm (Sorvall RC5B PLUS using an SS-34 rotor) for 15 min at 4˚C to pellet

252    intact cells and large cell debris and protein concentration in the supernatant was

253    determined using the Bio-Rad Protein Assay Reagent according to the manufacturer's

254    instructions. One microgram of total protein was separated by SDS-PAGE using 11%

255    acrylamide, 0.5% bis-acrylamide (Bio-Rad) gels and a Bio-Rad Mini-Protein Tetracell

256    chamber model 3000X1. Gels were run at 150 V until the dye front had moved

257    approximately 1 cm into the separating gel. Proteins in gels were stained with Instant Blue

258    (Expedeon) for 5 min and de-stained in water. The 1 cm of gel lane containing each sample

259    was cut out and proteins subjected to in-gel tryptic digestion using a DigestPro automated

260    digestion unit (Intavis Ltd). The resulting peptides were fractionated using an Ultimate 3000

261    nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific)

262    as previously described (25). The raw data files were processed and quantified using

263    Proteome Discoverer software v1.4 (ThermoScientific) and searched against the UniProt *P.*

264    *aeruginosa* PA01 database (5563 proteins; UniProt accession UP000002438), the *A.*

265    *baumannii* ATCC 17978 database (3783 proteins; UniProt accession UP0006737) or the *E.*

266    *coli* MG1655 database (4307 proteins; UniProt accession UP000000625). The database file is

267    provided as supplementary data. Proteomic searches against the databases was were

268    performed using the SEQUEST (Ver. 28 Rev. 13) algorithm. Protein Area measurements were

269    calculated from peptide peak areas using the "Top 3" method (26) and were then used to

270    calculate the relative abundance of each protein. Proteins with fewer than three peptide

271    hits were excluded from the analysis.

272    <u>Codon Usage Calculation</u>

273    The open reading frames of the 20 most highly expressed genes in each species were

274    downloaded from Genbank and concatenated into a single reading frame. The codon usage

275    calculator (https://www.biologicscorp.com/tools/CodonUsageCalculator#.WrZ02IjFI2w) was

276    applied to this concatenated open reading frame using the standard genetic code table.

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285

286 **Transparency Declaration**

287 None to declare – All authors.

288

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361        2006;5:144-156.

362    **Tables**

363    **Table 1:** The 20 most highly abundant proteins in *E. coli* during growth in Nutrient Broth

| Accession | Description | Gene Name | Mean Abundance (*n*=3) |
|---|---|---|---|
| P0CE48 | Elongation factor Tu | b3980 | 6.184E+08 |
| P0A9B2 | Glyceraldehyde-3-phosphate dehydrogenase A | b1779 | 3.399E+08 |
| P69776 | Major outer membrane lipoprotein Lpp | b1677 | 2.654E+08 |
| P0A7J3 | 50S ribosomal protein L10 | b3985 | 2.539E+08 |
| P06996 | Outer membrane protein C | b2215 | 2.425E+08 |
| P02359 | 30S ribosomal protein S7 | b3341 | 2.357E+08 |
| P0A7R1 | 50S ribosomal protein L9 | b4203 | 2.253E+08 |
| P0A7L0 | 50S ribosomal protein L1 | b3984 | 2.219E+08 |
| P60438 | 50S ribosomal protein L3 | b3320 | 2.022E+08 |
| P0AG55 | 50S ribosomal protein L6 | b3305 | 1.825E+08 |
| P0A910 | Outer membrane protein A | b0957 | 1.780E+08 |
| P0A7W1 | 30S ribosomal protein S5 | b3303 | 1.752E+08 |
| P62399 | 50S ribosomal protein L5 | b3308 | 1.743E+08 |
| P0A6M8 | Elongation factor G | b3340 | 1.644E+08 |
| P0A7V8 | 30S ribosomal protein S4 | b3296 | 1.642E+08 |
| P0A7R5 | 30S ribosomal protein S10 | b3321 | 1.622E+08 |
| P0AG67 | 30S ribosomal protein S1 | b0911 | 1.541E+08 |
| P0A7X3 | 30S ribosomal protein S9 | b3230 | 1.486E+08 |
| P0A7J7 | 50S ribosomal protein L11 | b3983 | 1.434E+08 |
| P0A7K2 | 50S ribosomal protein L7/L12 | b3986 | 1.422E+08 |

364

365 **Table 2**: The 20 most highly abundant proteins in *P. aeruginosa* during growth in Nutrient

366 Broth

| Accession | Description | Gene Name | Mean Abundance (*n*=3) |
|---|---|---|---|
| P09591 | Elongation factor Tu | PA4265 | 1.215E+09 |
| P30718 | 60 kDa chaperonin | PA4385 | 8.267E+08 |
| Q9HWP9 | Uncharacterized protein | PA4132 | 5.857E+08 |
| Q9I2V5 | Aconitate hydratase B | PA1787 | 4.160E+08 |
| P13794 | Outer membrane porin F | PA1777 | 3.949E+08 |
| P05384 | DNA-binding protein HU-beta | PA1804 | 3.624E+08 |
| Q9HVC4 | 50S ribosomal protein L25 | PA4671 | 3.562E+08 |
| Q9HWC6 | 50S ribosomal protein L1 | PA4273 | 3.536E+08 |
| Q9HWE1 | 30S ribosomal protein S3 | PA4257 | 3.471E+08 |
| Q9HWE7 | 50S ribosomal protein L5 | PA4251 | 3.181E+08 |
| Q9HZ71 | 30S ribosomal protein S1 | PA3162 | 3.074E+08 |
| Q9HWD5 | 50S ribosomal protein L3 | PA4263 | 3.037E+08 |
| Q9HWD1 | 30S ribosomal protein S7 | PA4267 | 3.020E+08 |
| Q9HWD6 | 50S ribosomal protein L4 | PA4262 | 2.792E+08 |
| Q9HWD8 | 50S ribosomal protein L2 | PA4260 | 2.691E+08 |
| Q9HWF0 | 50S ribosomal protein L6 | PA4248 | 2.658E+08 |
| Q9HWC7 | 50S ribosomal protein L10 | PA4272 | 2.490E+08 |
| Q9HVL6 | 50S ribosomal protein L21 | PA4568 | 2.433E+08 |
| O82851 | Elongation factor Ts | PA3655 | 2.337E+08 |
| O52759 | 30S ribosomal protein S4 | PA4239 | 2.276E+08 |

367

19

368 **Table 3**: The 20 most highly abundant proteins in *A. baumannii* during growth in Nutrient

369 Broth

| Accession | Description | Gene Name | Mean Abundance (*n*=3) |
|---|---|---|---|
| B7I876 | Outer membrane protein A | AB57_3344 | 2.562E+08 |
| B7I359 | 50S ribosomal protein L7/L12 | AB57_0368 | 1.138E+08 |
| B2HZ92 | 50S ribosomal protein L18 | AB57_3514 | 9.635E+07 |
| B0VSP5 | 60 kDa chaperonin | AB57_06545 | 9.230E+07 |
| B7IB16 | Peroxiredoxin | AB57_1341 | 8.615E+07 |
| B7GW08 | 30S ribosomal protein S3 | AB57_3524 | 8.434E+07 |
| B7IA20 | 50S ribosomal protein L15 | AB57_3511 | 8.280E+07 |
| B7I3K0 | Elongation factor Ts | AB57_2755 | 8.274E+07 |
| B7H1K1 | 50S ribosomal protein L1 | AB57_0366 | 7.769E+07 |
| B7I6T2 | Succinyl-CoA ligase [ADP-forming] subunit beta | AB57_3123 | 7.753E+07 |
| B7IBC1 | 30S ribosomal protein S6 | AB57_2509 | 7.533E+07 |
| B7I1W2 | ATP synthase subunit alpha | AB57_0191 | 7.474E+07 |
| B7IBK5 | Chaperone protein DnaK | AB57_0048 | 6.976E+07 |
| B7IA15 | 30S ribosomal protein S4 | AB57_3506 | 6.646E+07 |
| B7I1W4 | ATP synthase subunit beta | AB57_0193 | 6.254E+07 |
| B7I358 | 50S ribosomal protein L10 | AB57_0367 | 6.189E+07 |
| B0VQT3 | 50S ribosomal protein L6 | AB57_3515 | 6.138E+07 |
| B7IAS9 | 50S ribosomal protein L19 | AB57_3615 | 6.042E+07 |
| B7IA27 | 50S ribosomal protein L5 | AB57_3518 | 5.886E+07 |
| B7GYM8 | Elongation factor G | AB57_06485 | 5.795E+07 |

370

371     **Table 4:** Codon Usage Table for 20 Most Highly Expressed Genes in *E. coli*

```
TTT F   0.18  5.7 (    28)  TCT S  0.43 18.8 (    93)  TAT Y  0.19  5.0 (    25)  TGT C  0.37  1.4 (     7)
TTC F   0.82 25.8 (   128)  TCC S  0.35 15.1 (    75)  TAC Y  0.81 21.4 (   106)  TGC C  0.63  2.4 (    12)
TTA L   0.03  1.8 (     9)  TCA S  0.02  0.8 (     4)  TAA *  0.95  3.8 (    19)  TGA *  0.05  0.2 (     1)
TTG L(s) 0.03 1.8 (     9)  TCG S  0.00  0.2 (     1)  TAG *  0.00  0.0 (     0)  TGG W  1.00  6.7 (    33)

CTT L   0.03  2.2 (    11)  CCT P  0.09  3.0 (    15)  CAT H  0.26  3.8 (    19)  CGT R  0.71 40.5 (   201)
CTC L   0.03  2.4 (    12)  CCC P  0.01  0.4 (     2)  CAC H  0.74 10.7 (    53)  CGC R  0.28 16.1 (    80)
CTA L   0.00  0.0 (     0)  CCA P  0.14  4.6 (    23)  CAA Q  0.12  4.0 (    20)  CGA R  0.00  0.0 (     0)
CTG L(s) 0.88 63.3 (   314)  CCG P  0.76 25.6 (   127)  CAG Q  0.88 30.1 (   149)  CGG R  0.01  0.4 (     2)

ATT I   0.17  8.9 (    44)  ACT T  0.46 26.0 (   129)  AAT N  0.08  3.6 (    18)  AGT S  0.05  2.0 (    10)
ATC I   0.83 44.4 (   220)  ACC T  0.49 27.6 (   137)  AAC N  0.92 39.1 (   194)  AGC S  0.15  6.7 (    33)
ATA I   0.00  0.0 (     0)  ACA T  0.04  2.0 (    10)  AAA K  0.80 59.1 (   293)  AGA R  0.01  0.4 (     2)
ATG M(s) 1.00 24.0 (   119)  ACG T  0.02  1.0 (     5)  AAG K  0.20 14.7 (    73)  AGG R  0.00  0.0 (     0)

GTT V   0.57 55.1 (   273)  GCT A  0.51 54.5 (   270)  GAT D  0.28 16.5 (    82)  GGT G  0.59 53.2 (   264)
GTC V   0.06  5.8 (    29)  GCC A  0.07  7.5 (    37)  GAC D  0.72 42.0 (   208)  GGC G  0.40 35.7 (   177)
GTA V   0.28 27.0 (   134)  GCA A  0.25 27.0 (   134)  GAA E  0.81 56.9 (   282)  GGA G  0.00  0.4 (     2)
GTG V   0.09  9.1 (    45)  GCG A  0.16 17.4 (    86)  GAG E  0.19 13.3 (    66)  GGG G  0.01  0.8 (     4)
```

372

373     Codon usage for a concatenated sequence representing the open reading frames of the
374     genes encoding the 20 most abundant proteins, as shown in Table 1.
375     Values shown: Codon, Amino Acid, codon usage (fraction of 1), usage per 1000 in total
376     sequence, (absolute usage in total sequence).

377 **Table 5:** Codon Usage Table for 20 Most Highly Expressed Genes in *P. aeruginosa*

```
TTT F    0.08  2.1 (   11)  TCT S  0.04  1.9 (   10)  TAT Y  0.07  1.4 (    7)  TGT C  0.03  0.2 (    1)
TTC F    0.92 25.6 (  132)  TCC S  0.48 23.3 (  120)  TAC Y  0.93 18.8 (   97)  TGC C  0.97  5.2 (   27)
TTA L    0.00  0.2 (    1)  TCA S  0.00  0.0 (    0)  TAA *  0.75  2.9 (   15)  TGA *  0.20  0.8 (    4)
TTG L(s) 0.01  1.2 (    6)  TCG S  0.16  7.8 (   40)  TAG *  0.05  0.2 (    1)  TGG W  1.00  5.0 (   26)

CTT L    0.01  1.0 (    5)  CCT P  0.08  2.7 (   14)  CAT H  0.24  4.3 (   22)  CGT R  0.44 29.3 (  151)
CTC L    0.12  9.7 (   50)  CCC P  0.17  6.2 (   32)  CAC H  0.76 13.4 (   69)  CGC R  0.50 33.0 (  170)
CTA L    0.00  0.4 (    2)  CCA P  0.01  0.2 (    1)  CAA Q  0.15  5.2 (   27)  CGA R  0.01  0.6 (    3)
CTG L(s) 0.84 66.5 (  343)  CCG P  0.75 27.0 (  139)  CAG Q  0.85 29.3 (  151)  CGG R  0.04  2.7 (   14)

ATT I    0.09  4.8 (   25)  ACT T  0.12  5.2 (   27)  AAT N  0.08  2.5 (   13)  AGT S  0.02  1.2 (    6)
ATC I    0.91 48.3 (  249)  ACC T  0.85 37.1 (  191)  AAC N  0.92 30.5 (  157)  AGC S  0.30 14.4 (   74)
ATA I    0.00  0.0 (    0)  ACA T  0.01  0.4 (    2)  AAA K  0.24 17.1 (   88)  AGA R  0.00  0.2 (    1)
ATG M(s) 1.00 23.3 (  120)  ACG T  0.02  0.8 (    4)  AAG K  0.76 55.5 (  286)  AGG R  0.00  0.2 (    1)

GTT V    0.20 20.0 (  103)  GCT A  0.27 29.5 (  152)  GAT D  0.22 11.6 (   60)  GGT G  0.35 32.2 (  166)
GTC V    0.42 42.1 (  217)  GCC A  0.51 55.7 (  287)  GAC D  0.78 41.9 (  216)  GGC G  0.62 56.7 (  292)
GTA V    0.12 12.0 (   62)  GCA A  0.08  8.3 (   43)  GAA E  0.57 43.1 (  222)  GGA G  0.01  1.0 (    5)
GTG V    0.27 26.8 (  138)  GCG A  0.14 15.1 (   78)  GAG E  0.43 32.6 (  168)  GGG G  0.02  1.9 (   10)
```

378

379 Codon usage for a concatenated sequence representing the open reading frames of the
380 genes encoding the 20 most abundant proteins, as shown in Table 2.
381 Values shown: Codon, Amino Acid, codon usage (fraction of 1), usage per 1000 in total
382 sequence, (absolute usage in total sequence).

383 **Table 6:** Codon Usage Table for 20 Most Highly Expressed Genes in *A. baumannii*

```
TTT F      0.31  8.9 (    53)  TCT S   0.56 24.5 (   146)  TAT Y   0.46 10.9 (    65)  TGT C   0.80  3.4 (    20)
TTC F      0.69 20.1 (   120)  TCC S   0.01  0.5 (     3)  TAC Y   0.54 12.6 (    75)  TGC C   0.20  0.8 (     5)
TTA L      0.37 28.5 (   170)  TCA S   0.19  8.4 (    50)  TAA *   1.00  3.4 (    20)  TGA *   0.00  0.0 (     0)
TTG L(s)   0.19 14.9 (    89)  TCG S   0.05  2.2 (    13)  TAG *   0.00  0.0 (     0)  TGG W   1.00  3.7 (    22)

CTT L      0.37 28.5 (   170)  CCT P   0.35 11.9 (    71)  CAT H   0.16  1.7 (    10)  CGT R   0.88 46.5 (   277)
CTC L      0.02  1.3 (     8)  CCC P   0.00  0.0 (     0)  CAC H   0.84  8.9 (    53)  CGC R   0.11  5.7 (    34)
CTA L      0.03  2.4 (    14)  CCA P   0.55 18.6 (   111)  CAA Q   0.82 33.4 (   199)  CGA R   0.00  0.0 (     0)
CTG L(s)   0.02  1.2 (     7)  CCG P   0.09  3.2 (    19)  CAG Q   0.18  7.2 (    43)  CGG R   0.00  0.2 (     1)

ATT I      0.43 27.7 (   165)  ACT T   0.64 34.7 (   207)  AAT N   0.20  7.4 (    44)  AGT S   0.04  1.8 (    11)
ATC I      0.57 37.2 (   222)  ACC T   0.04  2.2 (    13)  AAC N   0.80 30.4 (   181)  AGC S   0.15  6.4 (    38)
ATA I      0.00  0.0 (     0)  ACA T   0.28 15.3 (    91)  AAA K   0.84 61.4 (   366)  AGA R   0.01  0.3 (     2)
ATG M(s)   1.00 24.0 (   143)  ACG T   0.03  1.7 (    10)  AAG K   0.16 11.9 (    71)  AGG R   0.00  0.0 (     0)

GTT V      0.55 47.5 (   283)  GCT A   0.54 63.9 (   381)  GAT D   0.44 24.8 (   148)  GGT G   0.80 69.1 (   412)
GTC V      0.03  2.9 (    17)  GCC A   0.02  1.8 (    11)  GAC D   0.56 31.4 (   187)  GGC G   0.18 15.4 (    92)
GTA V      0.35 30.2 (   180)  GCA A   0.28 33.0 (   197)  GAA E   0.84 65.7 (   392)  GGA G   0.02  1.3 (     8)
GTG V      0.07  5.7 (    34)  GCG A   0.16 18.8 (   112)  GAG E   0.16 12.7 (    76)  GGG G   0.00  0.3 (     2)
```

384

385 Codon usage for a concatenated sequence representing the open reading frames of the
386 genes encoding the 20 most abundant proteins, as shown in Table 3.
387 Values shown: Codon, Amino Acid, codon usage (fraction of 1), usage per 1000 in total
388 sequence, (absolute usage in total sequence).

389     **Table 7**: Primers used for Site Directed Mutagenesis

| Primer Name | Primer Sequence | Variant |
|---|---|---|
| a78g_F | 5'-CGCAGCAGAGTCTTTGCCAGATTTAAA**G**ATTGAAAAGCTTGAT-3' | M1 |
| a153g_F | 5'-GTGGGGCGTTGTTCCTAA**G**CATGGTTTGGTGG-3' | M2 |
| a216g_F | 5'-GACACTCCATTTACGGCTAA**G**GATACTGAAAAGTTAGTCAC-3' | M3 |
| a261g_a267g_F | 5'-TGGTTTGTGGAGCGTGGCTATAA**G**ATAAA**G**GGCAGCATTTCC-3' | M4,5 |
| a375g_a378g_F | 5'-CAAATGAACTGCTTAA**G**AA**G**GACGGTAAGGTTCAAGCCAC-3' | M6,7 |
| a435g_a441g_F | 5'-GGCTAGTTAA**G**AATAA**G**ATTGAAGTTTTTTATCCAGGCCCG-3' | M8,9 |
| a510g_F | 5'-GGTTTGGTTGCCTGAAAGGAA**G**ATATTATTCGGTGGTTGTTTT-3' | M10 |
| a537g_F | 5'-CGGTGGTTGTTTTATTAA**G**CCGTACGGTTTAGGCAATTTGG-3' | M11 |
| a597g_F | 5'-CTTGGCCAAAGTCCGCCAA**G**TTATTAAAGTCCAAATATGG-3' | M13 |
| a612g_F | 5'-CGCCAAGTTATTAAAGTCCAA**G**TATGGTAAGGCAAAACTGGTT-3' | M14 |
| a627g_F | 5'-CCAAGTATGGTAAGGCAAA**G**CTGGTTGTTCCAAGTCA-3' | M15 |
| a675g_F | 5'-GACGCATCACTCTTGAA**G**CTTACATTAGAGCAGGC-3' | M16 |
| a729g_F | 5'-GTTAAACGAAAGTAAAAAACCATCAAA**G**CCAAGCAACTAAATTTC-3' | M17 (KV) |

390

24

**Figure Legends**

**Figure 1: Impact of codon usage "optimisation" on IMP-1 enzyme activity in *E. coli*.**

IMP-1 enzyme activity was measured using imipenem as substrate in whole cell extracts of *E. coli* MG1655 carrying empty vector pRW50, or this vector with the wild-type (pHIMP) or codon-optimised (pHEcIMP) *bla*IMP-1 gene, each expressed from a hybrid strength integron promoter; or carrying a natural IMP-1 encoding plasmid originally from *P. aeruginosa*, which is the source of the cloned *bla*IMP-1 and upstream/promoter sequence (pNIMP). Data are means +/- Standard Deviation, *n*=4.

**Figure 2. Impact of AAA to AAG lysine codon conversion on IMP-1 protein production.**

IMP-1 protein abundance in clinical isolates of *P. aeruginosa*, *A. baumannii* and *E. coli* carrying the cloned wild-type *bla*IMP-1 gene (WT) and a variant having 17 AAA to AAG mutations (MUT). Protein abundance was measured in sonicated cell extracts and normalised using the abundance of the dominant selectable marker protein for the cloning vector carrying *bla*IMP-1. This was, for *E. coli*, where pSU18 was the cloning vector, Cat (chloramphenicol acetyl transferase) and for *P. aeruginosa* and *A. baumannii*, where pUBYT was the cloning vector, AphA (aminoglycoside [Kanamycin] phosphotransferase). Data are means +/- Standard Error of the Mean, *n*=3.

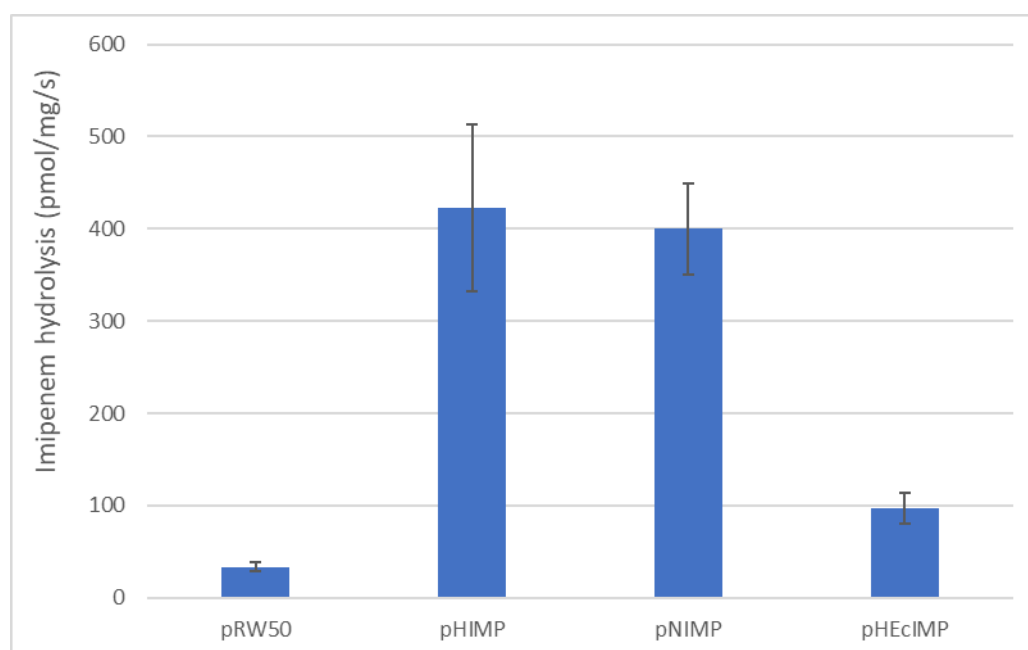**Figure 3. AAA to AAG lysine codon conversions in *bla*IMP-1**

The 17 AAA lysine codons converted to AAG are marked and sequentially numbered in the blaIMP-1 coding sequence. In some cases, two adjacent mutations were made at using a single primer in the same mutagenesis step, and are labelled as such: M x,y where x and y represent the two sequential mutations.

25

413 **Figure 4. Impact of sequential AAA to AAG lysine codon conversion on IMP-1 production in**

414 ***E. coli***

415 IMP-1 enzyme activity was measured using imipenem as substrate in whole cell extracts of

416 *E. coli* MG1655 carrying *bla*$_{IMP-1}$ cloned using pSU18 with one to seventeen AAA to AAG

417 mutations; each mutagenesis step being shown in figure 3. Some steps involved two

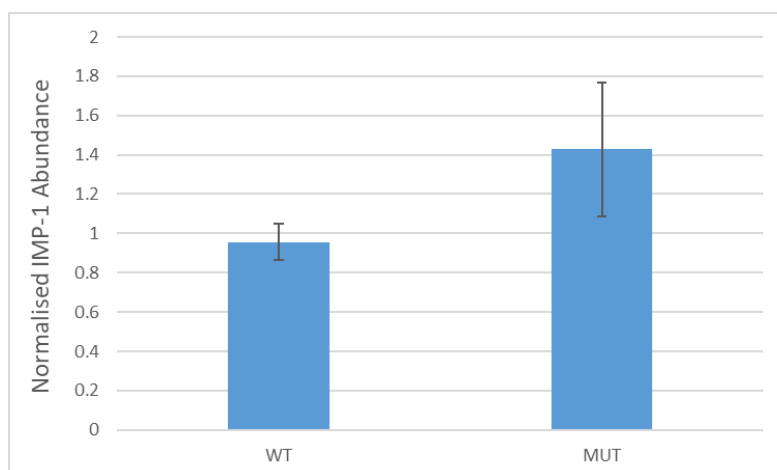418 adjacent mutations, e.g. M4,5. Data are means +/- Standard Deviation, *n*=4.
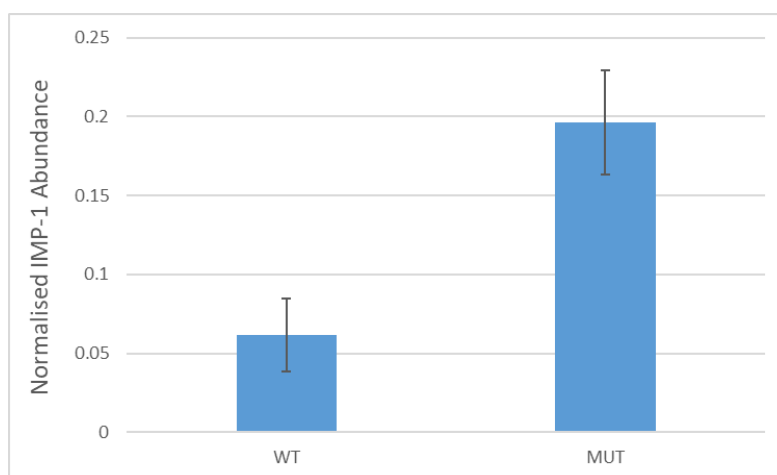
419

420    **Figure 1**
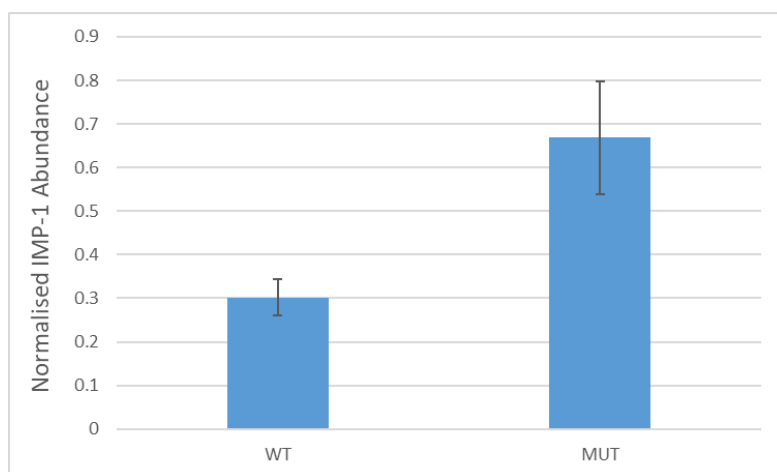


421

422 **Figure 2**

423 *P. aeruginosa.*



424

425 *A. baumannii*



426

427 *E. coli*



428

429 **Figure 3.**



430

431 **Figure 4.**

432



433