Phenotypic and genetic barriers to establishment of horizontally transferred genes encoding ribosomal protection proteins

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Background: Ribosomal protection proteins (RPPs) interact with bacterial ribosomes to prevent inhibition of protein synthesis by tetracycline. RPP genes have evolved from a common ancestor into at least 12 distinct classes and spread by horizontal genetic transfer into a wide range of bacteria. Many bacterial genera host RPP genes from multiple classes but tet(M) is the predominant RPP gene found in Escherichia coli.

Objectives: We asked whether phenotypic barriers (low-level resistance, high fitness cost) might constrain the fixation of other RPP genes in E. coli.

Methods: We expressed a diverse set of six different RPP genes in E. coli, including tet(M), and quantified tetracycline susceptibility and growth phenotypes as a function of expression level, and evolvability to overcome identified phenotypic barriers.

Results: The genes tet(M) and tet(Q) conferred high-level tetracycline resistance without reducing fitness; tet(O) and tet(W) conferred high-level resistance but significantly reduced growth fitness; tet(B(P)) conferred low-level resistance and while mutants conferring high-level resistance were selectable these had reduced growth fitness; otr(A) did not confer resistance and resistant mutants could not be selected. Evolution experiments suggested that codon usage patterns in tet(O) and tet(W), and transcriptional silencing associated with nucleotide composition in tet(B(P)), accounted for the observed phenotypic barriers.

Conclusions: With the exception of tet(Q), the data reveal significant phenotypic and genetic barriers to the fixation of additional RPP genes in E. coli.

Introduction

Tetracycline is a broad-spectrum natural antibiotic used to treat Gram-positive and Gram-negative bacterial infections.1 Tetracycline binds to the A site of the bacterial ribosome and inhibits protein synthesis by blocking binding of the incoming ternary complex. There are three clinically important mechanisms of acquired resistance to tetracycline: drug efflux; drug inactivation; and, the subject of this paper, ribosomal protection.2 Each mechanism involves the acquisition by horizontal genetic transfer (HGT) of genes encoding the relevant proteins. Based on the degree of similarity in their encoded protein sequences, ribosomal protection protein (RPP) genes are classified as a monophyletic family that has diverged into at least 12 distinct gene classes: tet(M), tet(O), tet(S), tet(W), tet(32), tet(Q), tet(T), tet(36), otr(A), tet(B(P)), tet and tet(44).2,3 A recent in silico analysis of genomic and metagenomic databases identified 116 distinct families of putative RPP genes.6

Six of these were expressed from a plasmid vector in Escherichia coli, of which one gave a strong tetracycline resistance phenotype, two had a marginal effect on resistance and three gave no resistance.6 The mechanism of resistance has been characterized for two RPP proteins, Tet(M) and Tet(O). Each protects the ribosome by displacing tetracycline from the A site, thereby allowing the ternary complex to bind and protein synthesis to continue.7-9

The tet(M) gene (Table S1, available as Supplementary data at JAC Online), found in at least 78 species, is the most widely distributed RPP gene, possibly due to its presence on the broad-host-range conjugative transposons Tn916 and Tn1545.2,5,10 Among the other RPP genes, tet(O), tet(W) and tet(Q) are also found in a wide range of both Gram-negative and Gram-positive bacterial species.5 Interestingly, tet(M) is the only RPP found repeatedly in E. coli isolates, including those from both human and animal sources,11-17 with only a single report (based on PCR fragment size) of tet(W).18 Given the evidence that many bacterial genera host...
different RPP genes,\textsuperscript{5} we asked whether phenotypic barriers might limit the spread of RPP genes other than tet(M) into \textit{E. coli}.

We addressed this question by genetically engineering six different genes (Table S1), representing the phylogenetic breadth of RPP proteins (Figure S1) into \textit{E. coli} and quantifying their tetracycline resistance and relative fitness phenotypes as a function of expression level. We also asked whether and how phenotypic barriers, if they existed, might be overcome and allow additional RPP genes to establish in a novel genetic environment.

**Materials and methods**

**Bacterial growth, antibiotics and susceptibility testing**

Bacteria were grown in LB with agitation (200 rpm), or on solid agar, LA (LB containing 1.5% agar; Oxoid, Basingstoke, UK) at 37°C or 30°C. Exponential growth rates were calculated from the rate of increase in OD as a function of time using a Bioscreen C (Oy Growth Curves, Finland). Antibiotics were from Sigma–Aldrich (Stockholm, Sweden) and used at the following final concentrations: tetracycline, 15 mg/L; chloramphenicol, 30 mg/L; kanamycin, 50 mg/L; and ampicillin, 100 mg/L. MIC was measured by broth microdilution using Mueller–Hinton II broth (Difco Becton, Dickinson, MD, USA) in round-bottomed 96-well plates, incubated at 37°C for 16–20 h before reading. MIC values are based on at least three independent measurements for each strain.

**Strain constructions**

Six RPP genes (Table S1) recombined into strains isogenic to \textit{E. coli} MG1655 were expressed from each of eight different constitutive promoters at the galK locus (Table S2). Strains were constructed as follows. The cat-sacB-SYFP2 cassette (chloramphenicol resistance, sucrose sensitivity, yellow fluorescence) was amplified using Phusion polymerase from CH3794 (prior to use; Sigma–Aldrich). Strains for transformation were from Sigma–Aldrich (Stockholm, Sweden) and used at the following final concentrations: tetracycline, 15 mg/L; chloramphenicol, 30 mg/L; kanamycin, 50 mg/L; and ampicillin, 100 mg/L. MIC was measured by broth microdilution using Mueller–Hinton II broth (Difco Becton, Dickinson, MD, USA) in round-bottomed 96-well plates, incubated at 37°C for 16–20 h before reading. MIC values are based on at least three independent measurements for each strain.

**PCR and local DNA sequencing**

Oligonucleotides (Sigma–Aldrich) for PCR and DNA sequencing are listed in Table S3. PCR products for recombining were amplified using Phusion\textsuperscript{9} High-Fidelity DNA Polymerase (New England Biolabs, MA, USA) in an S1000 Thermal Cycler (Bio-Rad) and were purified using the QAquick PCR Purification Kit (Qiagen, Hilden, Germany). PCR products for DNA sequencing were amplified using Fermentas 2× PCR Master Mix (Thermo Scientific, MA, USA). DNA sequencing of PCR products was performed at Macrogen Europe (Amsterdam, The Netherlands) or Eurofins Genomics using the MiSeq\textsuperscript{10} kit. Sequence reads were analysed using CLC Main Workbench 8.1.3 (CLCbio, Qiagen, Denmark).

**WGS**

Genomic DNA was isolated using the MasterPure DNA Purification Kit (Epicentre, Illumina Inc., WI, USA), resuspended in EB buffer, and concentrations determined using a Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific). DNA was diluted to 0.2 ng/mL in water and the samples were prepared for WGS according to the Nextera XT DNA Library Preparation Guide (Illumina Inc.). Samples were validated for DNA fragment size distribution using the Agilent High Sensitivity D1000 Screen Tape System (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed using a MiSeq\textsuperscript{18} desktop sequencer, according to the manufacturer’s instructions (Illumina Inc.). The sequencing data were aligned and analysed in CLC Genomics Workbench version 8.1.3.

**Protein expression measurements**

Relative protein expression levels were quantified using a MACSQuant VYB based on fluorescence intensity of translationally fused SYFP2 from 1.4 μL of overnight LB cultured mixed with 198.6 μL of 1× PBS. Mean fluorescence values relative to a non-fluorescent \textit{E. coli} MG1655 were based on at least four independent biological replicates.

**Growth competition experiments**

Strains for competition experiments, labelled with \textit{btuR::SYFP2-kan} and \textit{btuR::dTomato-kan}, were grown in four independent 2 mL LB cultures for 16 h at 37°C. Strains were mixed by adding 1 μL of each culture to 2 mL of LB and grown for 24 h at 37°C. The next day, 1.4 μL of each mixture was re-inoculated into 2 mL LB for another 24 h growth period at 37°C. In addition, 1.4 μL of each mixture was passaged to wells of a 96-well plate containing 198.6 μL of 1× PBS every 24 h for three consecutive days for fluorescence measurement (MACSQuant VYB). From each sample, 10 000 cells were counted and the ratio of SYFP2:Tomato-expressing cells was determined and used to calculate the selection coefficient.\textsuperscript{23,24} Selection coefficients were based on eight independent experiments (four competitions with one fluorescent pair and an identical set of four competitions with the fluorescent cassettes switched).

**Experimental evolution**

Ten independent cultures of CH6094, galK::J23100-tet(O) and CH6481, galK::J23100-tet(W) were grown from separate colonies in 2 mL of LB at 37°C overnight. From each culture, 2 μL was transferred into 2 mL LB containing tetracycline (16 μg/mL, to maintain selection for expression of resistance) and grown overnight at 37°C, resulting in 10 generations of net growth. Cultures were passaged for 50 successive passages (500 generations). After 500 generations of evolution, cultures were streaked on LA containing 16 μg/mL tetracycline and clones from each lineage were stored in LB with 15% glycerol at −80°C. One clone from each of the 10 evolved lineages of CH6094 and CH6481 was analysed for growth rate, tetracycline MIC and by WGS.

**Selection of mutants with reduced susceptibility to tetracycline**

Ten independent cultures of CH6489, galK::J23100-tetB(P) and CH6505, galK::J23100-otr(A), were grown in 2 mL LB at 37°C overnight. One hundred...
mircrolitres of each culture was plated onto LA containing tetracycline at concentrations 2-, 4-, 8- and 16-fold the MIC of the respective strains. Plates were incubated at 37°C for 48 h and the number of colonies recorded at 24 h and 48 h. From each lineage up to four colonies were picked and re-streaked twice on agar with the same tetracycline concentration. Mutant clones were assayed for growth rate, tetracycline MIC and by WGS.

Statistical analysis

Statistical analysis was performed using Excel Data Analysis ToolPak with relative gene expression as the independent variable and strain growth rate as the dependent variable. Regression analysis was performed and the significance F value was reported if less than 0.05.

Results and discussion

MIC dependence on protein expression levels

Six RPP genes, tet(M), tet(O), tet(W), tet(Q), tetB(P) and otr(A), chosen for phylogenetic diversity (Figure S1), were each engineered into the E. coli chromosome under the control of eight different constitutive promoters of varying strengths (Figure S2). Tetracycline MIC and relative protein expression level was measured for each gene (Figure 1, Table S4). The MIC increased up to 128 mg/L (256-fold increase over susceptible E. coli) as a function of increased levels. The expression level required to achieve this high MIC was lowest for tet(M), with tet(O), tet(Q) and tet(W) requiring higher expression levels to achieve the same MIC. There was a small expression-level-dependent increase in MIC associated with tetB(P) but expression peaked at a low level and the MIC did not exceed 8 mg/L. For otr(A), there was no increase in MIC despite high protein expression levels. We concluded that in addition to tet(M), expression of three other RPP genes not normally found in E. coli, tet(O), tet(W) and tet(Q), could generate high-level resistance to tetracycline, albeit requiring a higher level of protein expression.

Effect of expression levels on growth rate

We next asked whether the level of expression of the different RPP proteins affected bacterial fitness. We measured the exponential growth rate in rich medium and plotted this as a function of relative protein expression levels for each gene (Figure 2, Table S4). Growth rates decreased as a function of increased expression of tet(O) and tet(W) but for the other genes increased expression did not significantly affect the growth rate (Figure 2).

Evolving increased growth rates of tet(O)- and tet(W)-expressing strains

MIC and growth rate measurements identified tet(O) and tet(W) as RPP genes that could confer high-level resistance to tetracycline but with a significant fitness cost as a function of expression level. We asked whether and by what mechanism these fitness costs could be ameliorated. Strains expressing tet(O) and tet(W) at the highest level were experimentally evolved to select mutants with increased growth rates. After 500 generations of evolution, the relative growth rates of the tet(O)-expressing strains increased from 0.89 by an average of 5.5%, still below that of the control strain (Table S5). Of the evolved clones, 8/10 retained their tetracycline MIC at 128 mg/L. A common feature in 6/8 mutants was a mutation in or close to argW or proL (Table S5), encoding tRNAs that read AGG, a very rarely used codon in E. coli (Table S6). The growth rates of the evolved tet(W) strains increased from 0.81 by an average of 9.1%, still below that of the control strain (Table S7). Their original tetracycline MIC of 128 mg/L was maintained by 6/10 strains. Interestingly, all 10 clones had acquired a mutation in a tRNA gene, proL, encoding a tRNA that reads the relatively rarely used proline codon CCC (Table S6).

Phenotypes of argW and proL mutations associated with tet(O) and tet(W)

To test directly whether mutations in argW or proL were sufficient to account for the increases in growth rate observed in evolved tet(O) or tet(W), one mutation of each type was reconstructed in the unveled parental strains and evaluated for tetracycline MIC and growth rate (Table 1). The argW G41T mutation in combination with tet(O) maintained the high tetracycline MIC of the parental strain and increased the exponential growth rate by ~4%. The proL AntG7-G19 mutation in combination with tet(W) also maintained the high MIC of the parental strain and increased the growth rate from 0.81 to 0.91 relative to the control strain (Table 1).

We conclude that the mutations selected in the argW and proL tRNA genes are sufficient to explain the increased growth rates observed in the evolved strains expressing tet(O) and tet(W), respectively. We hypothesize that the mechanism of action by which the mutations in argW and proL increase the relative growth rates of strains expressing tet(O) and tet(W) is by decreasing the expression levels of the respective RPP proteins, thus reducing fitness cost. This is supported by the observation that strains with intermediate level expression have a tetracycline MIC of 128 mg/L, but
Figure 2. Exponential growth rate as a function of RPP expression level. Growth rates are displayed relative to the lowest expressing isogenic strain for each gene: (a) tet(M); (b) tet(O); (c) tet(W); (d) tet(Q); (e) tetB(P); and (f) otr(A). Linear regression was performed using the Excel Analysis ToolPak command Regression Analysis. * indicates significance F values of regression <0.05, a significant reduction in growth rate correlated with increased level of RPP expression.

Table 1. Phenotypes of constructed and evolved strains

| Strain   | Genotype                        | Tetracycline MIC (mg/L) | Relative growth rate (± SD) |
|----------|---------------------------------|-------------------------|-----------------------------|
| CH6098   | galK::J23113-tet(O)             | 0.5                     | 1.00 (0.02)*                |
| CH6094   | galK::J23100-tet(O)             | 128                     | 0.90 (0.02)                 |
| CH9243   | galK::J23100-tet(O), argW ntG41T | 128                     | 0.94 (0.02)                 |
| CH9619   | galK::J23100-tet(O), E. coli optimized | 128                     | 0.86 (0.02)                 |
| CH6485   | galK::J23113-tet(W)             | 0.5                     | 1.00 (0.02)*                |
| CH6481   | galK::J23100-tet(W)             | 128                     | 0.81 (0.03)                 |
| CH9244   | galK::J23100-tet(W), proL ΔntG7-G19 | 128                     | 0.91 (0.02)*                |
| CH9621   | galK::J23100-tet(W), E. coli optimized | 128                     | 0.90 (0.03)*                |
| CH6493   | galK::J23113-tetB(P)            | 0.5                     | 1.00 (0.04)                 |
| CH6489   | galK::J23100-tetB(P)            | 8                       | 0.93 (0.06)                 |
| CH9240   | galK::J23100-tetB(P), J23100 ntC26T | 32                     | 0.76 (0.12)                 |
| CH9241   | galK::J23100-tetB(P), J23100 ntG28C | 32                     | 0.73 (0.09)                 |
| CH9242   | galK::J23100-tetB(P), J23100 ntG30A | 32                     | 0.75 (0.03)*                |
| CH9245   | galK::J23100-tetB(P), Δhns       | 128                     | 0.88 (0.02)                 |
| CH9618   | galK::J23100-tetB(P), E. coli optimized | 128                     | 0.83 (0.03)                 |

MIC values are based on at least three independent assays for each strain. Growth rates, each based on at least four biologically independent measurements, are relative to the unevolved strain expressing the tet genes from the weakest promoter, J23113. *indicates statistically significant difference (P < 0.05) in growth rate relative to the isogenic strain expressing the respective tet-RPP gene at the highest level (two-tailed t-test).
with smaller reductions in growth rate (Figures 1 and 2, Table S4). As a further test of the hypothesis, strains were constructed with SYFP2 (yellow fluorescence) translationally fused to the tet genes. We observed that argW G41T and prol ∆ntG7-G19 (Figure 3) significantly decreased the translational level of Tet(O) and Tet(W) proteins, respectively. This is consistent with the hypothesis that mutating these tRNA genes can reduce costly expression of Tet(O) and Tet(W) RPP proteins, increasing the growth rate without reducing the tetracycline MIC, although full restoration of growth rate was not achieved (Table 1, Tables S5 and S7).

A general hypothesis is that high-level expression of tet(O) or tet(W) creates an excessive demand for rare tRNAs, trapping ribosomes unproductively and slowing growth rate. To test this hypothesis we synthesized codon-optimized genes under the control of the highest expression promoter and measured MIC and growth rate (Table 1). Codon optimization significantly increased growth rate with tet(W) but not tet(O). This supports the hypothesis for tet(W) but suggests that factors other than codon usage, for example high levels of Tet(O) might inhibit ribosome function, are additionally involved in determining the fitness costs associated with expression of tet(O).

**Selection for increased MIC in tetB(P) and otr(A)-expressing strains**

We addressed why no MIC increase was associated with high-level expression of otr(A) and why expression of tetB(P) was apparently restricted (Figure 1). We selected for resistant mutants using 10 independent cultures each of CH6505, galK::J23100-otr(A) and CH6489, galK::J23100-tetB(P) plated at four different tetracycline concentrations (see Materials and methods). No colonies were obtained with strains carrying otr(A) even at 2× MIC, suggesting there is no easy mutational path leading to a resistance phenotype for this gene in E. coli. To exclude the possibility that the native DNA sequence of otr(A) prevented phenotypic expression, we synthesized an E. coli-optimized version of the gene with regard to both GC:AT content and codon usage (CH9617, Table S1) and remeasured MIC when expressed from the strong J23100 promoter. We observed no increase in MIC from the E. coli-optimized otr(A) gene.

In contrast, with tetB(P), resistant mutants were obtained from all 10 cultures at each level of selection. We focused on mutants selected at the highest level, 8× MIC, which arose at a frequency of ~10⁻⁷. Thirty clones, distributed across 10 independent cultures, were analysed by WGS, and tetracycline MIC and growth rate were measured (Table S8). For each mutant, the growth rate was decreased relative to the parental strain (mutant mean 0.74, SD 0.12), while the tetracycline MIC increased from 8 to 32 mg/L (13 clones), 8 to 64 mg/L (11 clones) or 8 to 128 mg/L (6 clones). WGS analysis (Table S8) revealed that 14/30 mutants had acquired a mutation in hns, a gene encoding a histone-like DNA-binding protein with a preference for AT-rich sequences. Most of the hns mutations are predicted to cause inactivation: IS element in the coding sequence (11); amino acid substitution (2); or frameshift mutation (1). A further 6/30 mutants had acquired single nucleotide changes within the J23100 promoter, driving expression of tetB(P). Several mutants had acquired mutations affecting drug efflux regulators and transcriptional or translational regulation. Interestingly, half of the mutants (15/30) had acquired only a single mutation, 8 of which were knockout mutations in hns (Table S8). This strongly suggests that a knockout mutation in hns is sufficient to convert an E. coli with tetB(P) into a strain expressing a high level of resistance to tetracycline.

**Phenotypes of mutants with TetB(P) expressing tetracycline resistance**

To test whether mutations affecting the tetB(P) promoter, or inactivation of hns, were sufficient to account for the increased MIC (Table S8) we reconstructed these mutations in the unevolved parenteral strain. Three different single nucleotide changes in the promoter driving expression of tetB(P) increased the tetracycline MIC from 8 to 32 mg/L but reduced growth rates further, by approximately 19% (Table 1). Deletion of hns increased the tetracycline MIC from 8 to 128 mg/L and also reduced the growth rate further, by ~5% (Table 1). Accordingly, these individual mutations are sufficient to account for the phenotypes of the selected mutants, with increased MIC but decreased growth rate. We hypothesized that the promoter and hns mutations might both act by allowing increased transcription of tetB(P). The hns gene encodes a DNA-binding protein with a preference for AT-rich sequences and its activity is strongly associated with transcriptional silencing of foreign genes. A plausible conclusion is that increased transcription of tetB(P) is sufficient to increase tetracycline MIC, but at the cost of further reducing bacterial growth rate. The nucleotide composition of the tetB(P) coding sequence is 68.3% A + T (Table S6), much higher than the average of approximately 50% for E. coli. This high AT content of tetB(P) is compatible with the gene being a target for transcriptional silencing, which is relieved by mutations inactivating hns (Table 1). To test the hypothesis, we synthesized tetB(P) with a 50% GCAT ratio and codon-optimized for expression in E. coli (CH9618, Table 1) and measured MIC and growth rate
when expressed from the strong J23100 promoter. Sequence optimization of tetB(P) was sufficient to increase the MIC from 8 to 128 mg/L, but also reduced the growth rate by 11% relative to the non-optimized strain. These phenotypes are very similar to those caused by inactivation of hns and support the hypothesis that the activity of tetB(P) in E. coli is inhibited by having an AT-rich sequence that is subject to transcriptional silencing. Overall, these experiments explain the low MIC associated with tetB(P) in E. coli and show that selection to increase MIC is strongly associated with genetic changes that reduce growth rate.

**Conclusions**

This study has identified specific genetic and phenotypic barriers to the establishment of several foreign RPP genes in E. coli. We showed that the use of rare codons by tet(Q) and tet(W) contributed to reduced growth rate as a function of expression level, while high AT content was associated with transcriptional silencing of tetB(P) resulting in a low MIC. Expression of otr(A) did not result in any resistance to tetracycline and we were unable to select resistant mutants, suggesting that the problem might be at the level of protein function. Importantly, we identified one foreign RPP gene, tet(Q), which when expressed at a level approximately three times that of tet(M) resulted in high-level resistance to tetracycline without reducing growth rate (Figures 1 and 2, Table S4). Although tet(Q) has not yet been found in E. coli, it is frequently found in species of the mammalian gut microbiota such as Capnocytophaga, Bacteroides, Fusobacterium, Mitsuokella, Neisseria, Porphyromonas and Prevotella species. A potential HGT mechanism for tet(Q) has been described in Bacteroides, where it is situated within CTnDOT, a conjugative transposon. Although there is no evidence of tet(Q) being transferred via CTnDOT from Bacteroides to E. coli, an 18 kb fragment of this transposon not including tet(Q) was shown to be self-transmissible from Bacteroides to E. coli. There is also evidence of intraspecies transfer of tet(Q) between Bacteroides and Prevotella, based on sequence identity and transfer of tet(Q) between different oral Prevotella species. Our data on resistance and fitness suggest that there is potential for additional RPP genes like tet(Q) to establish in E. coli subject to the restrictions of HGT.

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**Transparency declarations**

None to declare.

**Supplementary data**

Tables S1 to S8 and Figures S1 and S2 are available as Supplementary data at JAC Online.
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