Overcoming codon-usage bias in heterologous protein expression in *Streptococcus gordonii*

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One of the limitations facing the development of *Streptococcus gordonii* into a successful vaccine vector is the inability of this bacterium to express high levels of heterologous proteins. In the present study, we have identified 12 codons deemed as rare codons in *S. gordonii* and seven other streptococcal species. tRNA genes encoding 10 of the 12 rare codons were cloned into a plasmid. The plasmid was transformed into strains of *S. gordonii* expressing the fusion protein SpaP/S1, the anti-complement receptor 1 (CR1) single-chain variable fragment (scFv) antibody, or the *Toxoplasma gondii* cyclophilin C18 protein. These three heterologous proteins contained high percentages of amino acids encoded by rare codons. The results showed that the production of SpaP/S1, anti-CR1 scFv and C18 increased by 2.7-, 120- and 10-fold, respectively, over the control strains. In contrast, the production of the streptococcal SpaP protein without the pertussis toxin S1 fragment was not affected by tRNA gene supplementation, indicating that the increased production of SpaP/S1 protein was due to the ability to overcome the limitation caused by rare codons required for the S1 fragment. The increase in anti-CR1 scFv production was also observed in *Streptococcus mutans* following tRNA gene supplementation. Collectively, the findings in the present study demonstrate for the first time, to the best of our knowledge, that codon-usage bias exists in *Streptococcus* spp. and the limitation of heterologous protein expression caused by codon-usage bias can be overcome by tRNA supplementation.

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INTRODUCTION

Streptococcus gordonii, a commensal bacterium of the human oral cavity, has gained interest as a live oral vaccine vehicle (Lee, 2003; Medaglini *et al.*, 1997). One of the major limitations facing the development of *Streptococcus gordonii* into a successful live vaccine vector is the inability of this bacterium to express high levels of heterologous proteins. It is believed that a number of 'bottlenecks' exist that limit heterologous protein expression, including codon-usage bias, inefficient translocation and folding of proteins, and degradation by cell-wall-associated and extracellular proteases (Tjalsma *et al.*, 2004).

Codon-usage bias is one of the best understood and characterized bottlenecks (Gustafsson *et al.*, 2004). Bacteria have biases towards certain codons, using some more frequently than others. As a result, some tRNAs exist in very low quantities because the associated codons are rarely

used. When heterologous genes containing a high percentage of rare codons are being expressed, protein translation is stalled or terminated due to the lack of charged tRNAs, resulting in truncated proteins and in lower levels of heterologous protein expression (Baca & Hol, 2000; Kane, 1995). One way to circumvent codon-usage bias is to supplement the bacterium with tRNAs for the rare codons on plasmids (Brinkmann *et al.*, 1989; Hua *et al.*, 1994). This method has proved to be successful in *Escherichia coli*, as demonstrated by commercially available plasmids carrying tRNA genes for rare codons (e.g. pRARE2). There are many examples in the literature demonstrating the increased yield of foreign proteins in *E. coli* using this approach (Baca & Hol, 2000; Kim & Lee, 2006; Zdanovskiy & Zdanovskaia, 2000). Alternatively, expression of heterologous genes can be optimized by converting rare codons into more frequently used codons (Sorensen *et al.*, 2003). This conversion has significantly increased yields of human interleukin-18 (hIL-18) and a malaria candidate vaccine when expressed in *E. coli* (Li *et al.*, 2003; Zhou *et al.*, 2004).

Abbreviation: CR1, complement receptor 1; RT-PCR, reverse transcription PCR; scFv, single-chain variable fragment.

To our knowledge, the problem of codon-usage bias and the means to overcome it have not been reported for the genus *Streptococcus*. In the present study, we have identified 12 codons that are deemed to be rare in *Streptococcus* and demonstrated that the difficulty in expressing a number of heterologous proteins can be overcome by tRNA supplementation. This finding could have far-reaching implications in gene expression in the genus *Streptococcus* as many species are important in industry and medicine.

METHODS

Bacteria and growth conditions. *Streptococcus gordonii* RJM4 was constructed previously (Lee *et al.*, 1999). It carries the *spaP/s1* fusion gene on its chromosome. *spaP* encodes the major surface LPXTG protein SpaP (also named P1 or antigen I/II) from *Streptococcus mutans* (Lee *et al.*, 1989). *s1* encodes the N-terminal 179 aa fragment of pertussis toxin subunit S1 and is fused to the middle part of *spaP* (Lee *et al.*, 1999). *S. gordonii* SMI/II-4 carries *spaP* on its chromosome. This strain was constructed by transforming the suicide plasmid pSMI/II-4 into *S. gordonii* (*hpgG::tet*; Lee *et al.*, 2002). pSMI/II-4 was derived from pSMI/II-3 (Homonylo-McGavin & Lee, 1996) by deleting the 4.5 kb DNA fragment carrying the streptococcal origin of replication, but the plasmid still carries *spaP*. Thus, *S. gordonii* RJM4 and SMI/II-4 are similar in that they carry one copy of *spaP/s1* and *spaP* on the chromosome, respectively.

S. gordonii SecCR1 was constructed previously (Knight *et al.*, 2008). It carries the synthetic single-chain variable fragment (scFv) antibody against the complement receptor 1 (CR1) on the *E. coli*-*Streptococcus* shuttle vector pDL276. *S. gordonii* SecC18 was constructed in this study. DNA encoding *Toxoplasma gondii* cyclophilin 18 (C-18) protein was amplified by PCR from pKJ97 (High *et al.*, 1994) using the primer pair SL330 (5'-CCTGGCCAGGCGGCCGAAAATGCCGAGTCAGAAAG-3') and SL331 (5'-CCTGGCCGGCCTGGCCCTCCAACAACCAATGTCCGT-3') (*Sfi*I sites underlined). The 514 bp PCR fragment encoding mature C-18 was inserted into the *Sfi*I sites on pSecCR1 (Knight *et al.*, 2008), giving pSecC18. The plasmid was then transformed into *S. gordonii* (*hpgG::tet*), yielding *S. gordonii* SecC18. *S. mutans* SecCR1 was obtained by transforming pSecCR1 into *S. mutans* 834 (*spaP::tet*; Lee *et al.*, 1989).

S. gordonii and *S. mutans* were grown in TYG (1% tryptone, 0.5% yeast extract, 0.3% K₂HPO₄ and 0.2% glucose; all w/v) in a 5% CO₂ incubator at 37 °C. Kanamycin, spectinomycin and tetracycline were included in the medium when needed at 250, 250 and 10 µg ml⁻¹, respectively. Recombinant *E. coli* was grown aerobically with vigorous shaking at 37 °C in Luria-Bertani broth (1% tryptone, 0.5% yeast extract and 1% NaCl; all w/v) containing (µg ml⁻¹) of ampicillin (100), kanamycin (50) or spectinomycin (50).

Construction of pTRNA2. Fig. 1 depicts the steps in the construction of pTRNA2. The tRNA genes for AGG (*argW*), CGG (*argX*), CGA (*argN5*), AUA (*ileX*), CUA (*leuW*) and CCC (*proL*) were amplified by PCR as a 2968 bp DNA fragment from pRARE2 (Novagen) using the primers SL433/SL434 (Table 1). The PCR fragment also contained six other tRNA genes (*argU*, *metT*, *thrT*, *thrU*, *glyT* and *glyT*), which are not deemed to be rare-codon tRNA genes for *Streptococcus*. The PCR product was restricted with *Sfi*I and *Sma*I, and cloned into the same sites on the *E. coli*-*Streptococcus* shuttle vector pDL278 (LeBlanc *et al.*, 1992), creating pRARE2/278. The tRNA genes for CCG (*proK*), UCC (*serW*), UCG (*serU*) and CUG (*leuT*) were amplified by PCR from the chromosome of *E. coli* XL-1 Blue using primer pairs listed in Table 1. The PCR products for *proK*, *serW* and *serU* were restricted by *Eco*RI and *Hind*III, and ligated by T4 DNA ligase. The ligated DNA was

amplified by PCR using primers SL455/SL460, restricted with *Sal*I and *Bam*HI, and cloned into the same sites on pUC19, creating pTRNA-KWU. The *leuT* tRNA gene fragment also carried the structural gene (but not the promoter) for the *hisR* tRNA gene, and was first cloned into the *Hind*III and *Bam*HI sites on pBluescript, then excised from the plasmid as a *Kpn*I-*Bam*HI fragment and cloned into pTRNA-KWU. The *Sal*I-*Sma*I fragment carrying these four tRNA genes was then cloned into the *Sal*I-*Sma*I sites on pRARE2/278. The resulting plasmid was named pTRNA2, carrying the tRNA genes for 10 rare codons and 7 other tRNA genes on the *E. coli*-*Streptococcus* shuttle plasmid pDL278. The plasmid was maintained in *E. coli* XL-1 Blue.

RNA isolation and reverse transcription-PCR (RT-PCR). RNA was extracted using the method as described by Peterson *et al.* (2000) with modifications. *S. gordonii* SecCR1 carrying pTRNA2 or pDL278 was grown to mid-exponential phase (OD₆₀₀ ≈ 0.6) and cells were collected by centrifugation (14 000 g, 10 min). The cells (3.0 × 10¹⁰ c.f.u.) were resuspended in 500 µl diethyl pyrocarbonate (DEPC)-treated water. Five millilitres of phenol, which contained 0.1% (w/v) SDS and saturated with citric acid buffer (50 mM sodium citrate, 50 mM citric acid, pH 4.3 with citric acid), was added. The suspension was boiled for 10 min and then cooled on ice. The aqueous phase was separated by centrifugation (3000 g, 10 min), extracted once with 2 vols acidic phenol/chloroform (1:1; 3000 g, 7.5 min), and then with 2 vols chloroform (3000 g, 7.5 min). The RNA was precipitated with 2 vols isopropyl alcohol in the presence of 0.3 M sodium acetate. The precipitated RNA was collected by centrifugation (15 000 g, 20 min), washed with 75% ethanol, and dissolved in 50 µl DEPC-treated water. The RNA was then treated with 100 U RNase-free DNase I (Sigma-Aldrich) for 15 min at room temperature, extracted with acidic phenol/chloroform followed by chloroform and precipitated. The RNA was dissolved in DEPC-treated water.

Reverse transcription reactions were performed for 50 min at 42 °C, using 200 ng RNA, 200 ng random primers (Invitrogen) and 200 U SuperScript II reverse transcriptase (Invitrogen) in a total volume of 20 µl 1 × First Strand Buffer (Invitrogen) containing 10 mM dithiothreitol and 0.5 mM each dNTP. Mock transcription was carried out in parallel as a control using the same amount of RNA, but the reverse transcriptase was omitted. Following the reaction, the mixtures were heated for 15 min at 70 °C, cooled and treated with 20 U DNase-free RNase A (Sigma-Aldrich) and 2 U RNase H (Invitrogen) for 30 min at 37 °C.

PCR was carried out using 1 µl cDNA or materials from mock transcription, 20 pmol tRNA primers (Table 1) and 0.5 U *Taq* DNA polymerase in 50 µl reaction volumes containing 1.5 mM MgCl₂ and 0.2 mM each dNTP. PCR was performed under the following cycling parameters: 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 65 °C for 30 s and 72 °C for 15 s, followed by 72 °C for 5 min. The PCR products were analysed on a 2% agarose gel by electrophoresis.

SDS-PAGE and Western blotting. Following growth, bacterial cultures were standardized to an OD₆₀₀ of 1 and incubated with 5% (w/v) TCA on ice for 30 min. Samples were then centrifuged for 5 min at 10 000 g. Supernatants were discarded and the pellet was washed once with 1 ml cold acetone and air-dried. The pellets were resuspended in the sample buffer of Laemmli (1970), boiled for 5 min, and centrifuged. Proteins in the supernatants were analysed by SDS-PAGE on polyacrylamide gels using the buffer system of Laemmli (1970). Proteins were stained with Coomassie Blue R-250. For Western immunoblotting, proteins were transferred to nitrocellulose membranes (Towbin *et al.*, 1979). The SpaPS1 protein was detected by the anti-S1 monoclonal antibody A4 (1/4000; Halperin *et al.*, 1991). The SpaP protein was detected by the monoclonal antibody 4-10A (1/7000; Ayakawa *et al.*, 1987). The anti-CR1 scFv and C18 proteins were detected using the anti-HA tag monoclonal antibody (1/10 000; Sigma Aldrich). The secondary antibody used was a goat anti-mouse IgG

Fig. 1. Construction of ptRNA2. The solid bar on pRARE2/278 indicates the 2968 bp DNA fragment carrying six rare tRNA genes (*argW*, *argX*, *argN5*, *ileX*, *leuW* and *proL*) in addition to six other tRNA genes (*argU*, *metT*, *thrT*, *thrU*, *glyT* and *glyT*). See Methods for details. pBS, pBluescript.

alkaline phosphatase conjugated antibody (1/20 000; Sigma-Aldrich). The intensity of the full-length 195 kDa SpaP/S1, 185 kDa SpaP, 31 kDa anti-CR1 scFv and 21 kDa C18 immunoreactive bands was estimated from three independent experiments by using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The results were analysed by Student's *t*-test for two-sample equal variant populations with two-tailed distribution, and a *P*-value of <0.05 was considered significant.

RESULTS

Identification of rare codons in *Streptococcus*

Examination of codon usage in *S. gordonii* revealed that 12 codons occurred at a low frequency (≤ 15 per thousand)

(Table 2). These 12 codons were also used at low frequencies in seven other *Streptococcus* species with two exceptions: CUG and CCG in *S. sanguinis*, and AUA in *S. agalactiae* were used at high frequency. In *E. coli*, 9 of the 12 codons showed a similar low usage, but GCG, CUG and CCG were used at high frequency.

Construction of a rare-codon tRNA plasmid

To facilitate the attempt to overcome the limitation imposed by codon-usage bias when expressing heterologous proteins in *Streptococcus*, a plasmid carrying 17 tRNA genes was constructed on the *E. coli*–*Streptococcus* shuttle plasmid pDL278. Fig. 1 depicts the construction of this plasmid, named ptRNA2. The plasmid carries the tRNA genes for 10

Table 1. Primers used for PCR amplification of tRNA genes from *E. coli* and pRARE2, or cDNA of tRNA from ptRNA2

tRNA (codon)	Primer	Sequence (5'–3') and restriction sites (underlined)	Comments
	SL433	ACCGAGCTCGGATCCACTAGT, <i>Sst</i> I	PCR product is 2968 bp, containing 12 tRNA genes: <i>argW</i> , <i>argX</i> , <i>argN5</i> , <i>ileX</i> , <i>leuW</i> , <i>proL</i> , <i>metT</i> , <i>thrT</i> , <i>thrU</i> , <i>glyT</i> , <i>glyT</i> and <i>argU</i>
<i>proK</i> (CCG)	SL434	AAGCCCGGGCTGGCGATTGAGGTTTCATC, <i>Sma</i> I	PCR product is 280 bp of the <i>proK</i> tRNA gene
	SL455	ATGGTCGACCAACAACCTGGTGTCACATC, <i>Sal</i> I	
<i>serW</i> (UCC)	SL456	CGGAATTCTCTCATCCTCATGAGCTGC, <i>Eco</i> RI	PCR product is 330 bp of the <i>serW</i> tRNA gene
	SL457	ACGAATTCGGAAGTATAAGTCCGTAAC, <i>Eco</i> RI	
<i>serU</i> (UCG)	SL458	ATAAGCTTCGCCCACCATGTTCACTG, <i>Hind</i> III	PCR product is 380 bp of the <i>serU</i> tRNA gene
	SL459	CGAAGCTTAATTGCTTTGTTCTCTGTTCC, <i>Hind</i> III	
<i>leuT</i> (CUG)	SL460	CGGATCCTGACGATCTAACCCCTCAAG, <i>Bam</i> HI	PCR product is 270 bp of the <i>leuT</i> tRNA gene
	SL461	CGGGATCCGGTGGTAGTAATACCGCGT, <i>Bam</i> HI	
	SL462	TATAAGCTTAGCTGCGCTACTCGCCGA, <i>Hind</i> III	
RT-PCR primer			Size of PCR product (bp)
<i>proK</i> (CCG)	SL672	CGGTGATTGGCGCAGCCTGGTAGC	77
	SL673	GAGGATTCGAACCTCCGACCCCTT	
<i>serW</i> (UCC)	SL674	GGTGAGGTGCCGAGTGGCTGAAGT	88
	SL675	GGCGGTGAGGGGGGGATTCTGAAG	
<i>serU</i> (UCG)	SL676	GGAGAGATGCCGGAGCGGCTGAAC	90
	SL677	TGGCGGAGAGAGGGGGGATTTGAAC	
<i>leuT</i> (CUG)	SL678	GCGAAGGTGGCGGAATTGGTAGAC	87
	SL679	TGGTGCAGGGGGGGGACTTGAAC	
<i>proL</i> (CCC)	SL680	CGGCACGTAGCGCAGCCTGGTAGC	77
	SL681	TGGTCGGCACGAGAGGATTGAACT	
<i>leuW</i> (CUA)	SL682	GCGGGAGTGGCGAAATTGGTAGAC	85
	SL683	TGGTGCAGGGAGGCGAGACTTGAAC	
<i>ileX</i> (AUA)	SL684	GGCCCCTTAGCTCAGTGGTTAGAG	76
	SL685	TGGTGGCCCCTGCTGGACTTGAAC	
<i>argX</i> (CGG)	SL686	GCGCCCGTAGCTCAGCTGGATAGAG	77
	SL687	TGGCGCGCCCGACAGGATTCTGAAC	
<i>argW</i> (AGG)	SL688	GTCCTCTAGTTAAATGGATATAA	75
	SL689	TGGTGTCCCCTGCAGGAATCGAAC	
<i>argN5</i> (CGA)	SL690	CCGCCATTAGCTCATCGGGATAGA	77
	SL691	TGGACCGCCATCGGAGACTCGAAC	

rare codons (CGG, CGA, AGG, AUA, CUG, CUA, CCG, CCC, UCC and UCG), and 7 other tRNA genes that are not deemed to be rare tRNAs for *Streptococcus*.

Overcoming rare-codon-usage bias in *S. gordonii*

To test the ability of ptRNA2 to overcome the limitation of codon-usage bias in the expression of heterologous proteins in *Streptococcus*, the plasmid was transformed into *S. gordonii* RJM4, which carries a genetic fusion of the N-terminal 179-aa fragment of S1 subunit of pertussis toxin and the surface protein SpaP from *S. mutans* (Lee *et al.*, 1999). The level of expression of the SpaP/S1 fusion has been reported previously to be low (Lee *et al.*, 1999; Mallaley *et al.*, 2006). The S1 sequence contains 15.6 % (28 of 179 amino acids) rare codons, while the SpaP sequence has 3.7 % (58 of 1573 amino acids) rare codons (Table 3). ptRNA2 was also transformed into *S. gordonii* SMI/II-4, which carries only *spaP* without *s1*. The transformants were analysed for the expression of *spaP/s1* or *spaP* by Western blotting. As shown in Fig. 2, the expression of SpaP1/S1

increased by 2.7 ± 0.3 -fold (mean $\pm$ SD, $P < 0.005$) in *S. gordonii* RJM4 carrying ptRNA2 compared to *S. gordonii* RJM4 carrying the control plasmid pDL278 (a, b). In contrast, the level of SpaP expression by *S. gordonii* SMI/II-4 was not affected by ptRNA2 (Fig. 2c, d, $P > 0.1$).

To further demonstrate the ability of ptRNA2 to overcome rare-codon-usage bias, the expression of two other heterologous proteins in *S. gordonii* was tested. The first heterologous protein was an scFv antibody against CR1 (Knight *et al.*, 2008), and the second was cyclophilin C18 from *T. gondii* (High *et al.*, 1994). The anti-CR1 scFv and C18 sequences contain 14.0 % (41 of 293 amino acids) and 8.1 % (13 of 162 amino acids) rare codons, respectively (Table 3). *S. gordonii* SecCR1 carrying pDL278 produced a minute amount of anti-CR1 scFv (Fig. 3). Convincingly, when *S. gordonii* SecCR1 was transformed with ptRNA2, the yield of anti-CR1 scFv was increased by 120 ± 10 -fold (mean $\pm$ SD, $P < 0.001$). Similarly, ptRNA2 was able to increase the production of C18 by 10 ± 1 -fold (mean $\pm$ SD, $P < 0.01$) by *S. gordonii* SecC18.

Table 2. Rare codons in *Streptococcus*

Rare codons (expressed as frequency per 1000) identified in *Streptococcus* species. A rare codon is defined as one that has a frequency of <15 per thousand for a given amino acid. *E. coli* K-12 is included as a comparison. Data are taken from the completed genome sequences from TIGR (www.tigr.org). *S. sanguinis* and *S. gordonii* data are from the Los Alamos National Laboratory (www.oralgen.lanl.gov).

Amino acid (codon)	<i>S. gordonii</i> Challis	<i>S. mutans</i> UA159	<i>S. mitis</i> NCTC 12261	<i>S. sanguinis</i> SK36	<i>S. pneumoniae</i> TIGR4	<i>S. pyogenes</i> SF370	<i>S. agalactiae</i> NEM316	<i>S. thermophilus</i> LMG18311	<i>E. coli</i> K-12 MG1655
Ala (GCG)	11.0	7.97	10.33	12.7	10.70	8.87	8.43	9.23	33.7
Arg (CGG)	11.2	5.95	4.60	15.5	4.80	5.52	3.13	2.79	9.85
Arg (CGA)	13.8	9.76	11.45	11.5	11.74	12.19	12.70	10.36	6.47
Arg (AGG)	5.6	6.49	4.19	5.8	5.01	6.48	7.68	5.66	2.25
Ile (AUA)	9.7	10.22	7.54	7.9	9.91	10.27	16.66	9.31	7.33
Leu (CUG)	15.0	9.75	9.27	25.9	8.83	6.48	4.43	6.24	52.8
Leu (CUC)	10.9	7.48	12.90	1.9	12.15	7.43	6.20	11.22	11.0
Leu (CUA)	11.8	7.58	11.21	9.5	11.22	11.36	12.51	10.65	3.68
Pro (CCG)	13.1	8.31	8.59	19.5	8.92	8.24	8.31	7.56	23.3
Pro (CCC)	9.1	7.62	8.47	10.9	8.94	8.98	7.62	7.57	5.5
Ser (UCC)	9.3	7.99	8.09	12.1	7.81	7.38	5.58	6.67	8.6
Ser (TCG)	5.6	5.27	5.53	6.8	5.88	5.29	5.35	5.57	8.9

To provide further support for the result described above, the expression of the 10 rare-codon tRNA genes was examined by RT-PCR. As shown in Fig. 4, the fact that these 10 tRNAs were detected following reverse transcription strongly indicates that the genes were expressed in *S. gordonii*.

Overcoming codon-usage bias in *S. mutans*

To demonstrate that the effect of ptRNA2 was not limited to *S. gordonii*, ptRNA2 was transformed into *S. mutans* SecCR1. As shown in Fig. 5, *S. mutans* SecCR1 carrying

ptRNA2 produced 4.8 ± 0.5 -fold (mean $\pm$ SD, $P < 0.05$) more anti-CR1 scFv than *S. mutans* SecCR1 carrying the control plasmid pDL278.

DISCUSSION

In the present study, we have identified 12 codons that are used at low frequency in *S. gordonii*. In general, these 12 codons are also used at low frequency in seven other *Streptococcus* species, suggesting that this is a common feature among species of this genus. Nine of the 12 codons are also used at low frequency in *E. coli*, but the remaining

Table 3. Rare codons in heterologous proteins expressed in *S. gordonii*

The values show the number of rare codons (identified in Table 2) in the pertussis toxin S1 fragment from *Bordetella pertussis*, anti-CR1 scFv, cyclophilin C18 from *T. gondii*, and SpaP from *S. mutans*.

Amino acid (codon)	S1 (180 codons)	Anti-CR1 scFv (293 codons)	C18 (162 codons)	SpaP (1573 codons)
Ala (GCG)	3	2	3	16
Arg (CGG)	3	0	0	2
Arg (CGA)	1	0	2	1
Arg (AGG)	2	6	0	2
Ile (AUA)	0	2	0	1
Leu (CUG)	3	8	0	8
Leu (CUC)	3	7	3	7
Leu (CUA)	0	3	0	6
Pro (CCG)	3	3	2	31
Pro (CCC)	4	1	2	4
Ser (UCC)	4	8	0	5
Ser (UCG)	2	1	1	3
Total no. of rare codons (% of total codons)	28 (15.6)	41 (14.0)	13 (8.1)	58 (3.7)

Fig. 2. Overcoming codon-usage bias in the expression of SpaP/S1 in *S. gordonii*. (a) A 7.5% SDS-PAGE gel showing an equivalent amount of protein from *S. gordonii* RJM4 carrying pDL278 (lane 1) or ptRNA2 (lane 2). * indicates the 195 kDa SpaP/S1 protein clearly visible in lane 2. (b) Immunoblot of SpaP/S1 from the same samples as in (a). (c) A 7.5% SDS-PAGE gel of *S. gordonii* SMI/II-4 carrying pDL278 (lane 3) or ptRNA2 (lane 4). (d) Immunoblot of SpaP from the same samples as in (c). The low-molecular-mass immunoreactive bands in (c) and (d) are presumably degradation products, as observed previously (Homonylo-McGavin & Lee, 1996; Lee *et al.*, 1999). The blots and gels are representative of three independent experiments.

Fig. 3. Overcoming codon-usage bias in the expression of anti-CR1 scFv and cyclophilin C18 in *S. gordonii*. (a) A 10% SDS-PAGE gel showing equal amounts of protein from *S. gordonii* SecCR1 carrying pDL278 (lane 1) or ptRNA2 (lane 2), and *S. gordonii* SecC18 carrying pDL278 (lane 3) or ptRNA2 (lane 4). (b) Immunoblot of anti-CR1 scFv or C18 from the same samples as in (a). The blots and gels are representative of three independent experiments.

Fig. 4. Detection of the 10 rare-codon tRNAs in *S. gordonii* by reverse transcription. Odd-numbered lanes: PCR products from reverse-transcription reactions; even-numbered lanes: PCR from mock transcription reactions; lane M, 100 bp DNA marker.

three codons are not, highlighting the differences between *Streptococcus* and *E. coli*.

We were able to clone or subclone 10 rare-codon tRNA genes from *E. coli* and the commercial plasmid pRARE2. Transformation of the plasmid carrying the 10 tRNA genes into *S. gordonii* resulted in an increased production of three heterologous proteins, whose genes were from three different phylogenetic origins. The S1 fragment was from a Gram-negative bacterium, *Bordetella pertussis*, C18 was from a parasite, *T. gondii*, and anti-CR1 scFv was from a murine source. In addition, ptRNA2 also increased the production of anti-CR1 scFv in *S. mutans*. These results clearly indicate the utility of ptRNA2.

Fig. 5. Overcoming codon-usage bias in the expression of anti-CR1 scFv in *S. mutans*. (a) A 12.5% SDS-PAGE gel of proteins from *S. mutans* SecCR1 carrying pDL278 (lane 1) or ptRNA2 (lane 2). Arrow indicates the 31 kDa anti-CR1 scFv protein visible in lane 2. (b) Immunoblot of anti-CR1 scFv from the same samples as in (a). The blot and gel are representative of three independent experiments.

The results from the RT-PCR indicate that the rare tRNAs were expressed in *S. gordonii*, despite their *E. coli* origin. The increase in heterologous protein expression obtained suggests that at least some of the rare-codon tRNAs were functional. Further experimentation to fully investigate the functionality of these tRNAs is warranted.

The finding that SpaP1/S1, but not SpaP production, was increased by ptRNA2 indicates that the presence of the S1 fragment in SpaP was the cause of the limited expression of SpaP/S1. This result is in accordance with the fact that the S1 sequence and not the SpaP sequence contains a high proportion of rare codons. SpaP/S1 produced by *S. gordonii* RJM4 carrying ptRNA2 is clearly visible on the Coomassie-Blue-stained SDS-PAGE gel (Fig. 2). Judging from the relative intensity of other protein bands on the same gel, SpaP/S1 was produced to a level comparable with authentic proteins. This is also the case for anti-CR1 scFv produced by *S. mutans* SecCR1 carrying ptRNA2 (Fig. 5). These results indicate that tRNA supplementation can overcome the limitation imposed by rare codons in heterologous protein production to a level comparable with authentic protein production.

To date, the problem of codon-usage bias has only been described in *E. coli* (Gustafsson *et al.*, 2004; Kane, 1995). To the best of our knowledge, our study is the first to identify that such a problem also exists in a genus other than *Escherichia*. In addition, we have demonstrated that codon-usage bias can be overcome by tRNA supplementation. Because the genus *Streptococcus* contains many industrially important species, our findings will be useful for the improvement of the biotechnological potential of some of these species. For *S. gordonii*, the current finding now enables us to shift our focus to overcoming other factors that limit high levels of heterologous protein production in this bacterium.

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