Codon usage in the *Mycobacterium tuberculosis* complex

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The usage of alternative synonymous codons in *Mycobacterium tuberculosis* (and *M. bovis*) genes has been investigated. This species is a member of the high-G + C Gram-positive bacteria, with a genomic G + C content around 65 mol %. This G + C-richness is reflected in a strong bias towards C- and G-ending codons for every amino acid: overall, the G + C content at the third positions of codons is 83 %. However, there is significant variation in codon usage patterns among genes, which appears to be associated with gene expression level. From the variation among genes, putative optimal codons were identified for 15 amino acids. The degree of bias towards optimal codons in an *M. tuberculosis* gene is correlated with that in homologues from *Escherichia coli* and *Bacillus subtilis*. The set of selectively favoured codons seems to be quite highly conserved between *M. tuberculosis* and another high-G + C Gram-positive bacterium, *Corynebacterium glutamicum*, even though the genome and overall codon usage of the latter are much less G + C-rich.

Keywords: codon usage, *Mycobacterium tuberculosis*, molecular evolution, intein

INTRODUCTION

Analyses of patterns of synonymous codon usage can reveal fundamental features of molecular evolution, and provide information of practical application in molecular biology. Codon usage is influenced by mutational biases, upon which the effects of natural selection may be superimposed (for a review, see Sharp *et al.*, 1993). Codon usage patterns are best understood in the Gram-negative bacterium *Escherichia coli*, where it seems clear that certain codons – those best recognized by the most abundant tRNA species – are translationally optimal (Ikemura, 1981). These optimal codons are strongly 'preferred' in highly expressed genes, whereas in genes expressed at low levels codon usage is more uniform (Ikemura, 1981; Gouy & Gautier, 1982). Codon usage in the low-G + C Gram-positive species *Bacillus subtilis* is similar to that in *E. coli* insofar as there are certain optimal codons which are more strongly preferred in more highly expressed genes, but differs in two respects: for some amino acids the optimal codons are different from those in *E. coli*, presumably reflecting differences in the tRNA populations of the two species, and overall the strength of codon usage bias is weaker (Shields & Sharp, 1987; Sharp *et al.*, 1990).

In contrast, in some bacterial species there appears to be little difference between the codon usage profiles of genes expressed at high and low levels, and so little evidence that translational selection has been effective. Several such species have extreme genomic base composition, being either very A + T-rich, e.g. *Mycoplasma capricolum* (Ohkubo *et al.*, 1987), or very G + C-rich, e.g. *Micrococcus luteus* (Ohama *et al.*, 1990) and *Streptomyces* species (Wright & Bibb, 1992). In these cases, the mutational biases seem to swamp any selection for particular codons, although it is also possible that tRNA profiles have adapted so that many of the codons favoured by the mutational bias have become optimal (Shields, 1990). Differences in the strength and efficacy of selection for optimal codons could also result from differences in the ecological/population genetics of various species.

As yet, rather few other bacterial species have been examined in any detail with respect to their codon usage. To gain any insight, it is essential that sequences should be available for genes expressed at different levels, and it is usually necessary to subject the data to multivariate statistical analyses to characterize any heterogeneity among genes. Thus, while codon usage compilations have been made for a number of other species (e.g. Wada *et al.*, 1992), the variation among genes has not been examined. Here we investigate codon usage in *Mycobacterium tuberculosis*. The molecular biology of this species is of great interest, since it is a human pathogen. It is the causative

Abbreviations: CA, correspondence analysis; CAI, codon adaptation index; F_{op} , frequency of optimal codons; N_e , effective number of codons; RSCU, relative synonymous codon usage.

agent of tuberculosis, responsible for around 3 million deaths per year; among infectious diseases it is the leading cause of death in man (Bloom & Murray, 1992). There are two reasons why codon usage in *M. tuberculosis* may not show evidence of translationally selected bias. First, like *Micrococcus* and *Streptomyces*, it is a member of the high-G+C Gram-positive bacteria (Olsen *et al.*, 1994), although its genomic G+C content is rather lower than in these other species (about 65 mol%, compared to about 75 mol%). Second, *M. tuberculosis* multiplies almost exclusively in macrophages, with a doubling time of about 24 h: if codon selection were mediated by the need for efficient use of ribosomes during periods of rapid growth (Andersson & Kurland, 1990), such selection may not be effective in this species. Thus, it is particularly interesting to ask whether genes expressed at high and low levels in *M. tuberculosis* differ in their codon usage.

METHODS

Sequences. DNA sequences were taken from the GenBank/EMBL/DDBJ DNA sequence database (GenBank release 87). Database entries identified in their features tables as *Mycobacterium tuberculosis* and *M. bovis* protein-coding sequences were extracted using the ACNUC retrieval system (Gouy *et al.*, 1985). Searches of the database for homologous sequences were performed using the BLAST program (Altschul *et al.*, 1990). Sequence alignments were performed using CLUSTAL W (Thompson *et al.*, 1994).

For nomenclature, we have tried to follow that in a recent listing of *Mycobacterium* sequences (Bergh & Cole, 1994). In the case of antigen sequences, we have followed Young *et al.* (1992). For the sequences homologous to the *groE* operon, we have adopted the terminology of Coates *et al.* (1993).

Analyses. Codon usage in the *Mycobacterium* sequences was calculated using the program CODONS (Lloyd & Sharp, 1992). As well as numbers of each codon, relative synonymous codon usage (RSCU) values were calculated. The RSCU is the observed frequency of a codon divided by the frequency expected if all synonyms for that amino acid were used equally, and so RSCU values close to 1.0 indicate a lack of bias. RSCU values are useful in comparing codon usage among genes, or sets of genes, encoding proteins with different amino acid compositions.

A number of indices of codon usage bias were calculated for each gene, also using CODONS, as follows.

$GC3_s$: the frequency of use of G+C in synonymously variable third positions of codons (i.e. excluding Met, Trp and termination codons). In addition, G+C values were calculated for each of the three codon positions, ignoring the distinction between silent and replacement sites.

N_c : the 'effective number of codons' used in a gene (Wright, 1990). This is a general measure of bias away from equal usage of alternative synonyms. Values of N_c can range between 20 (in an extremely biased gene, where only one codon is used per amino acid) and 61 (where all synonyms are used with equal probability). However, as G+C content at silent sites increases above 50%, the expected value for N_c under random codon usage (that is, random except for the influence of G+C content) decreases. The expected value is approximately given by:

$$N_c = 2 + s + \{29/[s^2 + (1-s)^2]\}$$

where s is $GC3_s$. See Wright (1990) for details of these calculations. (The expression for the expected value of N_c was misprinted there; F. Wright, personal communication.)

F_{op} : the 'frequency of optimal codons' used in a gene (Ikemura, 1981). This is a species-specific measure of bias towards those particular codons which appear to be translationally optimal in the particular species. Optimal codons for *M. tuberculosis* were identified (see below) for 15 of the 18 amino acids where alternative synonyms exist. Two optimal codons were identified for Leu and Arg, and one for each of 13 other amino acids. The F_{op} is calculated as the number of occurrences of these 17 optimal codons, divided by the total number of occurrences of these 15 amino acids.

The major trends in codon usage among genes were investigated using correspondence analysis (Greenacre, 1984). This is the most commonly used multivariate statistical approach in codon usage analysis (see, for example, Grantham *et al.*, 1981; Holm, 1986; Medigue *et al.*, 1991). In essence, this method plots genes according to their synonymous codon usage in a 59-dimensional space, and then identifies the major trends as those axes through this multidimensional hyperspace which account for the largest fractions of variation among genes.

RESULTS

Selection of DNA sequences for analysis

Sequences from both *Mycobacterium tuberculosis* and *Mycobacterium bovis* were included in the analysis. These two species, along with *Mycobacterium microti* and *Mycobacterium africanum*, form the '*M. tuberculosis* complex', and are so closely related that it has been suggested that they are in fact a single species (reviewed by Grange *et al.*, 1983). This close relationship is borne out by comparison of homologous genes from *M. tuberculosis* and *M. bovis*, where very little sequence variation is seen: the chaperonin-60-2 (65 kDa antigen; antigen 2) gene (Shinnick, 1987; Thole *et al.*, 1987), the 19 kDa antigen (antigen 13) gene (Young *et al.*, 1988; Collins *et al.*, 1990), and the *rpsL* and partial *rpsG* genes (F. Silbaq & H. Bercovier, GenBank accession no. L25882; Nair *et al.*, 1993) are all identical in the two 'species'. Furthermore, the only differences in the *cpn10* (also known as *groES*, encoding chaperonin-10, the 10 kDa antigen; antigen 5) gene sequences (Yamaguchi *et al.*, 1988; Baird *et al.*, 1989) occur near the termination codon, and are such that the reading frame is changed in a region where the *M. tuberculosis* protein is identical to that from *Mycobacterium leprae*; since *M. leprae* is quite distantly related to *M. tuberculosis*, these differences most likely reflect errors in the *M. bovis* sequence. There are no *M. africanum* protein-coding sequences in the database, and the only one from *M. microti*, 408 bp within the 32 kDa antigen gene, is also identical to *M. tuberculosis* and *M. bovis* (H. Soini *et al.*, GenBank accession nos. Z33653–Z33656). In contrast, for example, homologous gene sequences from different natural isolates of *E. coli* differ at 1–2% of nucleotides (see, for example, Hall & Sharp, 1992).

Duplicate sequences were excluded from the analysis. A number of *M. tuberculosis* catalase-peroxidase gene (*katG*) sequences occur in the database; we have analysed a consensus sequence. In addition, a number of other sequences identified in the GenBank/EMBL DNA sequence database as potential coding sequences were excluded. The identification of accurate gene sequences in

M. tuberculosis is difficult because of its high-G + C content. First, this can lead to sequencing errors because of compressions in G + C-rich regions. Second, stop codons, which are A + T-rich, occur relatively rarely even in noncoding sequences, so that long open reading frames (ORFs) can occur by chance. Third, the first results of the *M. leprae* genome project (Honore *et al.*, 1993) have suggested a surprisingly low gene density in that species (only about 40% of 36.7 kb was clearly genic), even though its genome is not unusually large (about 2.8 Mb); this compares with 88% coding density in the 4.7 Mb genome of *E. coli* (Plunkett *et al.*, 1993). One line of evidence that an ORF is indeed a functional gene comes from conservation of the sequence, without frameshifts, and with an excess of changes at silent sites, in other species. In the case of *M. tuberculosis*, a number of sequences can be compared to homologous segments of the *M. leprae* genome, particularly as a result of the *M. leprae* genome project, from which 20 cosmids totalling approximately 770 kb have been deposited in the database (Honore *et al.*, 1993; D. R. Smith, GenBank accession nos L01095, L01263, L01536, L04666; D. R. Smith & K. Robison, GenBank accession nos U00010–U00023; H. Fsihi & S. T. Cole, GenBank accession no. Z46257). A large number of potential coding sequences were excluded because their putative products exhibited no homology to any known protein, because in an alignment with homologous sequences there were insertion/deletion events leading to frameshifts, because they were in substantial overlap with other ORFs more likely to be genes, or because their positional base composition statistics were anomalous (see below). A few specific examples are given below.

A 1434 bp sequence (O'Connor *et al.*, 1990) contains a 812 bp ORF encoding a 35 kDa antigen (antigen 9; Young *et al.*, 1992), but also a 816 bp ORF on the complementary strand. The two ORFs overlap by 684 bp and are in the same phase, i.e. the first codon positions of one are complementary to the third positions of the other. Such complementary ORFs are not uncommon, but are probably a simple consequence of the relative scarcity in real genes of the codons (UUA, UCA and CUA) complementary to stop codons (Sharp, 1985; Merino *et al.*, 1994). In comparison with a homologous sequence from *M. leprae* (cosmid B2235; D. R. Smith & K. Robison, GenBank accession no. U00019), most changes in the 812 bp ORF were silent, whereas most in the 816 bp ORF were not. The 816 bp ORF was excluded.

A 3 kb sequence (Leao *et al.*, 1995) contains a 1539 bp ORF encoding a phospholipase C homologue. A second smaller ORF, on the complementary strand, is annotated in the database as encoding the MTP40 protein, but lies completely within the large ORF. The two ORFs are in different phases, sharing third codon positions. Consequently both are G + C-rich at third positions, but the second codon positions of the MTP40 ORF are more G + C-rich (59%) than the first positions (53%), which is most unusual in *M. tuberculosis* genes (see below). Furthermore, within this region the phospholipase C protein has similarity with phospholipases from *Pseudomonas*

aeruginosa, while the MTP40 ORF is not conserved. The MTP40 ORF was excluded.

Database entries for putative genes encoding dihydrofolate reductase (A. H. Patki & J. W. Dale, GenBank accession no. X59271) and thymidylate synthase (A. H. Patki & J. W. Dale, GenBank accession no. X59273) each potentially encode products that showed no detectable homology (in BLAST searches) with dihydrofolate reductase and thymidylate synthase sequences from other species. Furthermore, the thymidylate synthase ORF is not conserved in a homologous region from *M. leprae* cosmid B2235, while the dihydrofolate reductase ORF has a third position G + C content of 60%, 16% lower than any of 34 genes analysed below, and 5% lower than at first positions in this ORF, which is also quite unusual (see below); both ORFs were excluded.

The *M. tuberculosis dnaJ* gene sequence (R. Lathigra *et al.*, GenBank accession no. X58406) is 33 codons shorter than that of *M. leprae*. This appears to be due to a frameshift near the 3' end: insertion of an additional G into a run of three Gs at codons 341–342 restores a reading frame with a termination codon at a homologous position to that of *M. leprae* and encoding an amino acid sequence well conserved between *M. leprae* and *Streptomyces coelicolor*. Sequence after codon 341 was excluded from this analysis.

By comparison with the *asd* sequence from *Mycobacterium smegmatis* (Cirillo *et al.*, 1994b), the *M. bovis asd* gene (Cirillo *et al.*, 1994b) appears to contain a frameshift, due to the deletion of two nucleotides in codons 312–313. Insertion of two nucleotides restores a reading frame in which 14/15 differences between *M. bovis* and *M. smegmatis* are silent; codons 312 and 313 of *asd* were excluded from the analysis.

By comparison with homologous sequences from *E. coli* and *B. subtilis*, the *M. bovis rpsK* sequence (E. Dubnau *et al.*, GenBank accession no. U15140) may contain as many as four frameshifts, and so was excluded. In the same database entry, there is an ORF ('ORFX') located between *rpmJ* and *rpsM*, and annotated as encoding a ribosomal protein. The putative product has no similarity to any database entry, and furthermore, there is no such ORF between *rpmJ* and *rpsM* in other bacteria (*B. subtilis*, *Lactococcus lactis*, *Chlamydia trachomatis* and *E. coli*). ORFX overlaps *rpsM* by 58 bp and has a base composition quite unlike any other *M. tuberculosis* gene (see below): the G + C content at synonymously variable third positions is only 64%, and second positions of codons are far more G + C-rich (72%) than first positions (45%). ORFX was excluded from our analysis.

The database contains the sequence of one *M. tuberculosis* cosmid sequence with 23 annotated ORFs (D. R. Smith & K. Robison, GenBank accession no. U00024). Since these putative genes were not characterized by a directed approach, and most do not have clear homologues in the database, these ORFs were excluded from our analysis.

Several problematic sequences putatively encode antigens. Consequently, we excluded from the analysis all sequences thought to encode antigens, unless these were

Table 1. *M. tuberculosis* gene sequences

Gene	Product	AA	GC	GC3 _s	N _c	F _{op}	Accession no.	Reference†
<i>rplL</i> *	Ribosomal protein L7/L12	130	0.68	0.92	25.1	0.84	D16310	1
<i>cpn10-1</i>	Chaperonin-10-1 (Ag 5; GroES)	100	0.64	0.88	33.5	0.70	M25258	2
<i>rpsL</i>	Ribosomal protein S12	124	0.63	0.81	38.0	0.65	L08011	3
<i>infA</i> *	Initiation factor 1	73	0.63	0.89	34.5	0.68	U15140	4
<i>cpn60-2</i>	Chaperonin-60-2 (Ag 2; GroEL)	540	0.65	0.87	31.5	0.73	M15467	5
<i>rpsS</i> *	Ribosomal protein S19	93	0.58	0.81	39.1	0.61	S65565	6
<i>tuf</i>	Elongation factor TU	396	0.64	0.88	30.8	0.72	X63539	7
<i>rpoC</i>	RNA polymerase beta'	291 +	0.64	0.88	34.1	0.66	U11452	8
<i>dnaK</i>	70 kDa heat-shock protein (Ag 1)	609	0.63	0.81	38.3	0.61	X58406	9
<i>recA</i>	RecA protein	350	0.64	0.84	35.1	0.59	X58485	10
<i>nrdE</i>	Ribonucleotide reductase	723	0.59	0.85	37.2	0.63	L34407	11
<i>rpsD</i> *	Ribosomal protein S4	132 -	0.63	0.80	35.5	0.63	U15140	4
<i>rpoB</i>	RNA polymerase beta	1177	0.64	0.86	34.5	0.64	L27989	12
<i>sodA</i>	Superoxide dismutase (Ag 4)	207	0.59	0.79	43.6	0.63	X52861	13
<i>gyrB</i>	DNA gyrase B	686	0.60	0.77	41.5	0.55	L27512	14
<i>cpn60-1</i>	Chaperonin-60-1	539	0.65	0.83	38.8	0.53	X60350	15
<i>rpsG</i> *	Ribosomal protein S7	156	0.63	0.78	43.7	0.57	L25882	16
<i>rpsM</i> *	Ribosomal protein S13	123	0.63	0.77	39.2	0.61	U15140	4
<i>katG</i>	Catalase-peroxidase	740	0.65	0.82	39.8	0.58	-	17
<i>gyrA</i>	DNA gyrase A	838	0.64	0.82	37.4	0.58	L27512	14
<i>adh*</i>	Alcohol dehydrogenase	346	0.65	0.87	34.2	0.59	X63450	18
<i>aroB</i>	3-Dehydroquinate synthase	362	0.69	0.87	34.2	0.60	X59509	19
<i>mtrA</i>	Two-component regulator	225	0.62	0.78	40.0	0.55	U01971	20
<i>inhA</i>	EnvM protein homologue	269	0.66	0.86	36.8	0.55	U02492	21
<i>polA</i>	DNA polymerase I	904	0.68	0.87	36.3	0.59	L11920	22
<i>grpE</i>	Heat-shock protein	235	0.66	0.76	39.8	0.50	X58406	9
<i>asd*</i>	Semialdehyde dehydrogenase	343 +	0.69	0.88	34.3	0.56	Z18290	23
<i>argF</i> *	Ornithine carbamoyltransferase	307	0.69	0.89	33.9	0.60	X64203	24
<i>rpmJ</i> *	Ribosomal protein L36	37	0.59	0.75	41.8	0.54	U15140	4
<i>aroQ</i>	3-Dehydroquinase	147	0.66	0.83	40.0	0.59	X59509	19
<i>pboS</i>	Phosphate binding protein (38 kDa; Ag 3)	374	0.65	0.82	45.4	0.53	M30046	25
<i>dapB</i> *	Dihydropicolinate reductase	271	0.71	0.87	34.2	0.53	L24366	26
<i>dnaJ</i>	Heat-shock protein	341 +	0.68	0.82	38.3	0.52	X58406	9
<i>mtrB</i>	Two-component sensor	567	0.67	0.81	41.5	0.54	U14909	27
<i>lysA</i>	Diaminopimelate decarboxylase	446	0.64	0.77	44.1	0.54	M94109	28
<i>bccA</i>	Biotin carboxyl carrier	654	0.68	0.80	41.7	0.48	Z19549	29
<i>ald</i>	L-Alanine dehydrogenase (Ag 6)	373	0.64	0.75	44.2	0.50	X63069	30
‡*	Mycocerosic acid synthase	2110	0.67	0.78	42.0	0.51	M95808	31
<i>plc</i>	Phospholipase C	512	0.63	0.79	43.3	0.52	L11868	32
<i>aroA</i>	EPSP synthase	450	0.71	0.82	38.1	0.46	M62708	33
<i>uraA</i> *	Orotidine-5'-monophosphate decarboxylase	274	0.73	0.85	40.5	0.46	U01072	34

* *M. bovis* sequence.

† References: 1, Ohara *et al.* (1993b); 2, Baird *et al.* (1989); 3, Nair *et al.* (1993); 4, E. Dubnau and others (unpublished); 5, Shinnick (1987); 6, Ohara *et al.* (1993a); 7, Carlin *et al.* (1992); 8, M. Rohrbach and others (unpublished); 9, R. Lathigra and others (unpublished); 10, Davis *et al.* (1991); 11, Yang *et al.* (1994); 12, Miller *et al.* (1994); 13, Zhang *et al.* (1991); 14, Takiiff *et al.* (1994); 15, Kong *et al.* (1993); 16, F. Silbaq & H. Berçovier (unpublished); 17, Cockerill *et al.* (1995); 18, Stelandre *et al.* (1992); 19, Garbe *et al.* (1991); 20, R. Curcic & V. Deretic (unpublished); 21, Banerjee *et al.* (1994); 22, Mizrahi *et al.* (1993); 23, Cirillo *et al.* (1994b); 24, Timm *et al.* (1992); 25, Andersen & Hansen (1989); 26, Cirillo *et al.* (1994a); 27, R. Curcic & V. Deretic (unpublished); 28, Andersen & Hansen (1993); 29, Norman *et al.* (1994); 30, Andersen *et al.* (1992); 31, Mathur & Kolattukudy (1992); 32, Leao *et al.* (1995); 33, Garbe *et al.* (1990); 34, Aldovini *et al.* (1993).

‡ No gene symbol used by authors.

Table 2. Codon usage in the 41 *M. tuberculosis* genes listed in Table 1

N number of codons; RSCU, relative synonymous codon usage; ter, termination codon.

AA	Codon	N	RSCU	AA	Codon	N	RSCU	AA	Codon	N	RSCU	AA	Codon	N	RSCU
Phe	UUU	69	0.28	Ser	UCU	32	0.21	Tyr	UAU	80	0.43	Cys	UGU	35	0.60
	UUC	417	1.72		UCC	203	1.33		UAC	294	1.57		UGC	82	1.40
Leu	UUA	18	0.07		UCA	54	0.35	ter	UAA	4	0.32	ter	UGA	22	1.74
	UUG	290	1.06		UCG	356	2.32	ter	UAG	12	0.95	Trp	UGG	172	—
Leu	CUU	64	0.23	Pro	CCU	49	0.22	His	CAU	96	0.51	Arg	CGU	154	0.76
	CUC	262	0.96		CCC	291	1.30		CAC	279	1.49		CGC	535	2.64
	CUA	68	0.25		CCA	64	0.29	Gln	CAA	116	0.40		CGA	79	0.39
	CUG	941	3.44		CCG	494	2.20		CAG	459	1.60		CGG	395	1.95
Ile	AUU	124	0.46	Thr	ACU	62	0.23	Asn	AAU	74	0.31	Ser	AGU	52	0.34
	AUC	668	2.46		ACC	682	2.58		AAC	410	1.69		AGC	222	1.45
	AUA	24	0.09		ACA	43	0.16	Lys	AAA	101	0.30	Arg	AGA	9	0.04
Met	AUG	321	—		ACG	270	1.02		AAG	563	1.70		AGG	43	0.21
Val	GUU	153	0.39	Ala	GCU	147	0.30	Asp	GAU	248	0.43	Gly	GGU	331	0.82
	GUC	618	1.57		GCC	937	1.89		GAC	911	1.57		GGC	854	2.12
	GUA	50	0.13		GCA	148	0.30	Glu	GAA	325	0.57		GGA	122	0.30
	GUG	750	1.91		GCG	754	1.52		GAG	806	1.43		GGG	304	0.75

clearly homologous to genes known in other species. One advantage of this approach is that for many of the remaining genes homologues have been sequenced from *E. coli* or *B. subtilis*, thus allowing comparisons of codon usage among these species. In addition, most partial sequences were excluded from the analysis. As a result, a total of 41 *M. tuberculosis* (including *M. bovis*) genes were included (Table 1).

Codon usage in *M. tuberculosis*

Overall codon usage across these 41 genes is presented in Table 2, which contains a total of 17612 codons. As expected, the overall G+C-richness of the *M. tuberculosis* genome (65%) is reflected in a strong bias towards C- and G-ending codons for every amino acid. Overall, the G+C content values for the first, second and third positions of codons are 67%, 45% and 83%, respectively. The rank order of these values is as expected in a G+C-rich genome (Osawa *et al.*, 1992). Furthermore, this rank order is consistent across individual genes. For example, the mean value for GC1–GC2 (where GCX is the G+C content at the Xth codon position) is 0.22, with a minimum of 0.09 (in *plc*). The mean value for GC3–GC1 is 0.16; only one gene (*grpE*, with a value of -0.004) has a value less than 0.05.

Nevertheless, there is some variation in codon usage patterns among genes (Table 1). This is reflected in GC3_s values, which range from 0.75 to 0.92 (mean 0.83), and in N_c values, which range from 25.1 to 45.4 (mean 37.9). Wright (1990) has suggested plotting N_c values against GC3_s as an approach to exploring codon usage variation

Fig. 1. Effective number of codons used (N_c) in each gene plotted against the G+C content at synonymously variable third positions of codons (GC3_s). The curve represents the expected value of N_c if bias is only due to G+C content. The genes represented by open symbols are described in the text.

among genes. For *M. tuberculosis* these values are highly (negatively) correlated (Fig. 1); the correlation coefficient, r , is -0.83 ($P < 0.0001$). However, such a correlation is expected with high (or low) GC3_s values. The curve in Fig. 1 shows the N_c values expected if codon usage is biased only by G+C content. Genes furthest away from (below) this curve are those whose codon usage is most biased in other respects. The genes represented by open

Fig. 2. Correspondence analysis of codon usage variation among *M. tuberculosis* genes. Genes are plotted at their coordinates on the first two axes produced by the analysis, and are listed in Table 1 in their order of appearance on axis 1, from left to right. The genes represented by open symbols to the left are *rplL*, *cpn10-1*, *rpsL*, *cpn60-2* and *tuf* (with high codon usage bias); those to the right are *plc*, *aroA* and *uraA* (with low bias).

symbols in Fig. 1 are (from left to right) those encoding ribosomal protein S4 (*rpsD*), the GroEL chaperonin homologue (*cpn60-2*), elongation factor Tu (*tuf*), and ribosomal protein L12 (*rplL*), which are all expected to be highly expressed. In fact, the two lowest values of N_c are for the *rplL* and *tuf* genes, which in *E. coli* at least, encode two of the most abundant proteins in the cell.

A more sophisticated approach to explore codon usage variation among genes is correspondence analysis, CA (Greenacre, 1984; Grantham *et al.*, 1981). CA of these 41 genes produced first and second axes accounting for 16% and 9% of the total variation, respectively. The fact that the variation along the first axis is nearly twice that for the second is suggestive that there may be a single source of systematic variation among genes. Genes have been plotted according to their positions on the first two axes in Fig. 2. In Table 1, genes are listed in order of their position on axis 1 produced by the CA. Interestingly, all of the genes at one end of axis 1 (at the left in Fig. 2, and at the top of Table 1) are those known or expected to be highly expressed, and which have strong codon usage bias in *E. coli* and *B. subtilis*. These tend to be the genes with stronger codon usage bias (as assessed by N_c values) in *M. tuberculosis*: position on axis 1 and N_c values are correlated ($r = 0.61$, $P < 0.001$). In addition, the genes at the other extreme of axis 1 are those known or expected to be expressed at low levels (e.g. *uraA* and *aroA*), and whose *E. coli* or *B. subtilis* homologues have low codon usage bias. Thus, it appears that the first axis produced by CA is differentiating among genes according to codon usage differences associated with gene expression level.

To elucidate the differences between genes at the two extremes of axis 1, we have compiled (Table 3) codon usage values for two subsets of genes: *tuf*, *rplL*, *rpsL*, *cpn10-1* and *cpn60-2* (representing the high bias/

expression extreme; total 1295 codons) and *aroA*, *plc* and *uraA* (representing the low bias/expression extreme; total 1613 codons); these genes are shown by open symbols in Fig. 2. Codon frequencies (within amino acids) in these two groups were contrasted using a heterogeneity chi-square test, taking $P < 0.01$ as the criterion of significance due to the large number of tests performed: 10 codons, for 10 different amino acids, are used significantly more often in the 'high' genes than in the 'low' genes. A further 7 codons are more frequently used, but give chi-square values with probabilities in the range 0.01–0.10. These include UUC, AUC and AAC, which have been found to be the optimal codons for Phe, Ile and Asn, respectively, in a wide range of species (Sharp *et al.*, 1988; Sharp & Devine, 1989). In each case, the codon is very heavily used in the 'high' group, but the chi-square value is not significant because of the relatively high usage in the 'low' group. As more sequences become available, these differences may become significant. We tentatively designate all 17 of these codons (for 15 amino acids) as 'optimal' codons, to calculate the frequency of optimal codons (F_{op}) in each gene, since using only the codons with significant values would cover only about half of the genetic code. (In Table 3, these 17 codons are indicated in bold, with * marking the 10 codons with $P < 0.01$.) In a gene with the same amino acid composition as the total data set (Table 2), and in which codon usage were simply determined by a G + C content at silent sites of 83%, the F_{op} value would be about 0.55. The observed F_{op} values range between 0.46 and 0.84. These values ought to be correlated with position on axis 1, and indeed the high value of this correlation (0.90) indicates that this is a reasonably succinct summary of the spread along this axis. No simple codon usage statistic was found to be correlated with the second or subsequent axes.

For 35 of the 41 genes chosen for our analysis, the sequence of a homologue is available from either *E. coli* or *B. subtilis* (or both). The strength of species-specific codon usage bias in the *E. coli* and *B. subtilis* genes has been estimated using the codon adaptation index (CAI; Sharp & Li, 1987; Shields & Sharp, 1987), and is compared to the F_{op} values for the *M. tuberculosis* genes in Table 4. *M. tuberculosis* expresses two homologues of GroEL, now called chaperonin 60 (Kong *et al.*, 1993). One gene lies in an operon with the *groES* homologue, and one is located elsewhere; these have been termed *cpn60-1* and *cpn60-2*, respectively (Coates *et al.*, 1993). A great deal of research has been directed at a 65 kDa antigen (antigen 2), which is the product of the *cpn60-2* gene, but not at the product of the *cpn60-1* gene. This may indicate that *cpn60-2* is more highly expressed than *cpn60-1*, and the F_{op} value for *cpn60-2* (0.73) is rather higher than that for *cpn60-1* (0.53). Since *groEL* has strong codon usage bias in *E. coli* and *B. subtilis*, this may indicate that *cpn60-2* rather than *cpn60-1* is the functional homologue of *groEL*, even though it is *cpn60-1* that is found in an operon with the *groES* homologue. Excluding *cpn60-1*, the correlation of CAI and F_{op} is 0.56 for *E. coli* (32 genes) and 0.61 for *B. subtilis* (24 genes). Considering only 22 genes that can be compared among all three species, the correlation

Table 3. Codon usage in *M. tuberculosis* genes with high and low expression levels

See text and Fig. 2 for further details.

AA	Codon	High		Low		AA	Codon	High		Low	
		N	RSCU	N	RSCU			N	RSCU	N	RSCU
Phe	UUU	1	0.08	10	0.40	Ser	UCU	1	0.15	1	0.07
Phe	UUC	25	1.92	40	1.60	Ser	UCC	17	2.62	20	1.40
Leu	UUA	0	0.00	4	0.15	Ser	UCA	0	0.00	12	0.84
Leu	UUG	8	0.44	37	1.39	Ser	UCG	13	2.00	30	2.09
Leu	CUU	2	0.11	8	0.30	Pro	CCU	4	0.31	5	0.16
Leu	CUC	22	1.21	19	0.71	Pro	CCC	15	1.18	49	1.59
Leu	CUA	1	0.06	9	0.34	Pro	CCA	4	0.31	11	0.36
Leu	CUG*	76	4.18	83	3.11	Pro	CCG	28	2.20	58	1.89
Ile	AUU	10	0.45	15	0.75	Thr	ACU	2	0.09	5	0.18
Ile	AUC	57	2.55	43	2.15	Thr	ACC*	75	3.26	68	2.47
Ile	AUA	0	0.00	2	0.10	Thr	ACA	3	0.13	6	0.22
Met	AUG	21	—	29	—	Thr	ACG	12	0.52	31	1.13
Val	GUU	17	0.49	11	0.32	Ala	GCU	12	0.33	14	0.25
Val	GUC	70	2.00	48	1.40	Ala	GCC*	84	2.30	96	1.73
Val	GUA	4	0.11	5	0.15	Ala	GCA	7	0.19	30	0.54
Val	GUG	49	1.40	73	2.13	Ala	GCG	43	1.18	82	1.48
Tyr	UAU	0	0.00	10	0.63	Cys	UGU	0	0.00	4	0.47
Tyr	UAC*	22	2.00	22	1.38	Cys	UGC	1	2.00	13	1.53
ter	UAA	0	—	0	—	ter	UGA	2	—	3	—
ter	UAG	3	—	1	—	Trp	UGG	3	—	22	—
His	CAU	0	0.00	11	0.73	Arg	CGU*	14	1.29	7	0.42
His	CAC*	15	2.00	19	1.27	Arg	CGC	35	3.23	37	2.24
Gln	CAA	2	0.11	15	0.60	Arg	CGA	1	0.09	10	0.61
Gln	CAG*	36	1.89	35	1.40	Arg	CGG	15	1.38	39	2.36
Asn	AAU	2	0.11	9	0.41	Ser	AGU	2	0.31	1	0.07
Asn	AAC	34	1.89	35	1.59	Ser	AGC	6	0.92	22	1.53
Lys	AAA	4	0.08	8	0.64	Arg	AGA	0	0.00	3	0.18
Lys	AAG*	100	1.92	17	1.36	Arg	AGG	0	0.00	3	0.18
Asp	GAU	11	0.29	16	0.39	Gly	GGU*	39	1.32	28	0.65
Asp	GAC	66	1.71	67	1.61	Gly	GGC	68	2.31	89	2.06
Glu	GAA	16	0.27	25	0.88	Gly	GGA	5	0.17	16	0.37
Glu	GAG*	104	1.73	32	1.12	Gly	GGG	6	0.20	40	0.92

coefficients are: 0.65 (*M. tuberculosis* vs *E. coli*), 0.58 (*M. tuberculosis* vs *B. subtilis*), and 0.65 (*E. coli* vs *B. subtilis*).

Codon usage in an intein

The *recA* gene of *M. tuberculosis* (Davis *et al.*, 1991) is interesting because it contains an insertion encoding an intein (Perler *et al.*, 1994), i.e. the primary translation product is subject to protein splicing. In our initial analysis the part of the sequence encoding this intein was excluded. Codon usage in the intein is different from that in the 'extein' (i.e. the codons for the mature RecA protein): the intein is less G + C-rich ($GC_3 = 0.70$), and has a lower F_{op} (0.50). Inteins appear to be mobile elements. For example, while the *M. leprae recA* also

contains an intein, it is highly divergent from that in *M. tuberculosis* and located at a different position (Davis *et al.*, 1994). Thus, the intein may not have been present in the *M. tuberculosis recA* gene long enough for its codon usage to be selected to as high a level of bias as in the extein.

DISCUSSION

M. tuberculosis genes predominantly use C- and G-ending codons, presumably because of a G + C-biased mutation pattern in this species. Nevertheless, codon usage varies among *M. tuberculosis* genes. The primary source of variation is in the use of a subset of codons, which we have tentatively concluded are the translationally optimal codons in this species, since their frequency appears to be

Table 4. Codon usage bias in homologues

<i>E. coli</i>		<i>B. subtilis</i> *		<i>M. tuberculosis</i>	
Gene	CAI	Gene	CAI	Gene	F_{op}
<i>rplL</i>	0.81	(<i>rplL</i> 0.80)		<i>rplL</i>	0.84
<i>groEL</i>	0.79	<i>groEL</i>	0.69	<i>cpn60-2</i>	0.73
<i>tufB</i>	0.79	—	—	<i>tuf</i>	0.72
<i>groES</i>	0.51	<i>groES</i>	0.63	<i>cpn10-1</i>	0.70
<i>infA</i>	0.48	<i>infA</i>	0.59	<i>infA</i>	0.68
<i>rpoC</i>	0.68	(<i>rpoC</i> 0.62)		<i>rpoC</i>	0.66
<i>rpsL</i>	0.66	—	—	<i>rpsL</i>	0.65
<i>rpoB</i>	0.63	<i>rpoB</i>	0.55	<i>rpoB</i>	0.64
<i>rpsD</i>	0.57	<i>rpsD</i>	0.62	<i>rpsD</i>	0.63
<i>sodA/B</i>	0.71/0.55	—	—	<i>sodA</i>	0.63
<i>rpsM</i>	0.45	<i>rpsM</i>	0.76	<i>rpsM</i>	0.61
<i>dnaK</i>	0.72	<i>dnaK</i>	0.66	<i>dnaK</i>	0.61
<i>rpsS</i>	0.46	<i>rpsS</i>	0.57	<i>rpsS</i>	0.61
<i>thrA</i>	0.31	<i>dapG</i>	0.40	<i>ask</i>	0.61
<i>aroB</i>	0.28	<i>aroB</i>	0.39	<i>aroB</i>	0.60
<i>argF</i>	0.36	<i>argF</i>	0.37	<i>argF</i>	0.60
<i>polA</i>	0.39	—	—	<i>polA</i>	0.59
<i>recA</i>	0.61	<i>recE</i>	0.49	<i>recA</i>	0.59
<i>murA</i>	0.32	—	—	<i>adh</i>	0.59
<i>gyrA</i>	0.52	<i>gyrA</i>	0.50	<i>gyrA</i>	0.58
<i>katG</i>	0.51	—	—	<i>katG</i>	0.58
<i>rpsG</i>	0.61	—	—	<i>rpsG</i>	0.57
<i>usg</i>	0.44	<i>asd</i>	0.51	<i>asd</i>	0.56
<i>envM</i>	0.60	—	—	<i>inhA</i>	0.55
<i>gyrB</i>	0.55	<i>gyrB</i>	0.47	<i>gyrB</i>	0.55
<i>rpmJ</i>	0.46	<i>rpmJ</i>	0.76	<i>rpmJ</i>	0.54
<i>lysA</i>	0.33	<i>lysA</i>	0.48	<i>lysA</i>	0.54
<i>groEL</i>	0.79	<i>groEL</i>	0.69	<i>cpn60-1</i>	0.53
<i>pboS</i>	0.58	—	—	<i>pboS</i>	0.53
<i>dapB</i>	0.34	<i>dapB</i>	0.48	<i>dapB</i>	0.53
<i>dnaJ</i>	0.51	<i>dnaJ</i>	0.49	<i>dnaJ</i>	0.52
—	—	<i>grpE</i>	0.46	<i>grpE</i>	0.50
—	—	<i>ald</i>	0.45	<i>ald</i>	0.50
<i>fabG</i>	0.56	—	—	<i>bccA</i>	0.48
<i>aroA</i>	0.36	<i>aroE</i>	0.43	<i>aroA</i>	0.46

* Values in parentheses indicate that the gene sequence is incomplete.

correlated with gene expression level. One indirect way to examine whether the F_{op} (frequency of optimal codons) values for *M. tuberculosis* relate well to gene expression level is to compare them to codon bias values for homologues from *E. coli* and *B. subtilis*, where the link between codon usage bias and gene expression level is quite well established. (This is making the assumption that many genes will be expressed at similar levels in different bacterial species.) The *M. tuberculosis* values are approximately as well correlated with CAI values in *E. coli* and *B. subtilis* as the latter two are with each other. We conclude that the differentiation in codon usage among *M. tuberculosis* genes is most likely due to selection for optimal codons being more effective on genes expressed at

higher levels. However, the extent of heterogeneity among genes in *M. tuberculosis* is rather lower than in *E. coli* and *B. subtilis*, which may reflect weaker selection in *M. tuberculosis* due to its long generation time.

Among the 17 codons identified above as likely to be translationally optimal, 15 end in C or G. Among these, UUC, UAC and AAC belong to two codon families read (in both *E. coli* and *B. subtilis*) by a single tRNA with guanosine or a modified guanosine in the first position of the anticodon. Such tRNAs can bind to U-ending codons using wobble interactions, but the standard Watson–Crick basepairing with a C-ending codon seems to be faster and more stable (Labuda *et al.*, 1982; Thomas *et al.*, 1988; Curran & Yarus, 1989). This would explain why these codons are preferred in a wide range of species. For other amino acids (e.g. Val and Ala), the preferred codons in *M. tuberculosis* differ from those in *E. coli* and *B. subtilis*. This presumably reflects a difference among these species in their tRNA populations. In the short term, codon usage is selected to match the tRNAs available. But in the long term, under sustained mutation pressure towards G + C (or A + T), tRNA populations should eventually adapt to match the most prevalent codons (Shields, 1990). Thus, persistent mutation bias towards G + C-richness in *M. tuberculosis* may have forced changes in the expression level of tRNAs, or even substitutions in the anticodons of tRNA genes. The exceptions to this trend are the U-ending codons for Arg and Gly; interestingly, these also appear to be the preferred codons in a wide range of species (Sharp *et al.*, 1988; Sharp & Devine, 1989).

To investigate how codon usage patterns evolve, it is necessary to compare more closely related species, i.e. to compare *M. tuberculosis* with other high-G + C Gram-positive bacteria. Honore *et al.* (1993) compiled codon usage values for the 12 ORFs in *M. leprae* cosmid B1790. The relative values are not dissimilar to those for *M. tuberculosis* (Table 2), even though the *M. leprae* genome is significantly more A + T-rich (57% G + C). They found a third codon position G + C content of 75% (compared to 83% here for *M. tuberculosis*). Given the 8% difference in genomic G + C content, third positions of codons would be expected to differ by rather more than 8% between the two species. This anomaly would appear to be due to the particular genes present in the *M. leprae* cosmid. Five encode ribosomal proteins, two encode elongation factors, and two encode RNA polymerase subunits; thus, nine of the 12 ORFs are expected to be highly expressed. By simply asking how many of the 61 sense codons were not used in each gene, Honore *et al.* (1993) found evidence that the degree of bias varied among the ORFs in the cosmid. Thus, the compiled codon usage for these 12 ORFs may be somewhat atypical, and a more random set of genes may be less G + C-rich at silent sites.

The genera *Nocardia* and *Corynebacterium* are quite closely related to *Mycobacterium* (Olsen *et al.*, 1994). Coque *et al.* (1993) examined codon usage in a small data set (14 ORFs) from *Nocardia lactamdurans*. This species appears to be very G + C-rich: although genomic G + C content was not given, for 27.6 kb of sequence analysed the value was

70%. GC3_s values were found to be very high and to only vary between 0.91 and 0.97. Thus, *N. lactamdurans* seems to be similar to *Micrococcus luteus* (Ohama *et al.*, 1990) and *Streptomyces* species (Wright & Bibb, 1992), where codon usage varies little among genes and is largely dominated by G + C-richness.

Codon usage in *Corynebacterium glutamicum* (genomic G + C content 53%) has been examined in a little more detail. Eikmanns (1992) found more biased codon usage values in a set of five highly expressed genes (mainly encoding glycolytic enzymes) than in 15 genes involved in amino acid biosynthesis and expected to be expressed at only moderate levels. Malumbres *et al.* (1993) combined data from *C. glutamicum* and *Brevibacterium lactofermentum* (which they showed from sequence comparisons to be extremely closely related, if not the same species) to yield a data set of 34 genes, among which seven were represented twice (i.e. both the *C. glutamicum* and the *B. lactofermentum* copies were included). The 12% difference in genomic G + C content between *C. glutamicum* and *M. tuberculosis* is reflected in overall codon usage: the average GC3_s among the 34 *C. glutamicum* genes is 0.62 (Malumbres *et al.*, 1993), compared to 0.83 for *M. tuberculosis*. Eikmanns (1992) and Malumbres *et al.* (1993) both defined the 'preferred' codons for *C. glutamicum*, but neither took account of whether the codon was significantly more frequently used in highly expressed genes. However, all but one of the 17 codons designated here as optimal in *M. tuberculosis* appear among the 'preferred' codons in *C. glutamicum*. The exception is GCC: the preferred codon(s) for Ala in *C. glutamicum* is GCA (and perhaps GCU). In addition, for Pro the preferred codon(s) in *C. glutamicum* is CCA (and perhaps CCU); while we did not designate an optimal codon for Pro, neither CCA nor CCU is used at high frequency in highly expressed *M. tuberculosis* genes (Table 3). In both cases, the difference is consistent with the divergence in genomic G + C content between the two species. Overall, the subset of synonymous codons which appear to be selected for in the two species has diverged very little.

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