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Codon Usage Bias Prefers AT Bases in Coding Sequences Among the Essential Genes of *Haemophilus influenzae*

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Abstract

The base composition at three different codon positions in relation to codon usage bias and gene expressivity was studied in a sample of twenty five essential genes from *Haemophilus influenzae*. ENC, CBI and Fop were used to quantify the variation in codon usage bias for the cds. CAI is used to estimate the level of gene expression of the cds selected in the present study. To find out the relationship between the extent of codon bias and nucleotide composition the values of A, T, G, C and GC they were compared with the A3, T3, G3, C3 and GC3 values, respectively. The results showed relatively weak codon usage bias among the coding sequences (cds) of *Haemophilus influenzae*. This in turn, implies that the essential genes prefer to use a set of restricted codons. However, the base compositional analysis of essential genes in *Haemophilus influenzae* revealed preference of AT to GC bases within their coding sequences and this preference might affect gene expression as indicated by the relatively high CAI values of the coding sequences.

Keywords: *codon usage pattern, gene expression, synonymous codons*

Introduction

The genetic information from mRNA is passed to protein with the help of translational machinery, a fundamental process occurring within all living cells. The genetic code uses sixty-four codons to encode the proteins. Many codons are redundant *i.e.* two or more codons code for a single amino acid, with the exception of methionine (AUG) and tryptophan (UGG). Such codons are described as being synonymous, and mostly differ by one nucleotide at the third codon position. Codon usage bias (CUB) refers to the non-random usage of synonymous codons for encoding the same amino acid in a protein (Lu *et al.*, 2005). In general, synonymous codons are used in unequal frequencies between genomes, genes from the same genome and within a single gene (Hooper and Berg, 2000; Lavner and Kotler, 2005; Supek and Vlahovicek, 2005). The most frequently used codons are termed as optimal or major codons, recognized by the most abundant tRNA species (Butt *et al.*, 2014). The least frequently used codons are non optimal or minor codons and lead to the premature termination during elongation stage of translation. Thus, CUB is accounted for by the positive selection for the optimal codons and the negative selection against the non optimal codons (Qin *et al.*, 2004). CUB is a unique property, that shows species-specific deviation (Grantham *et al.*, 1981). Thus the understanding of the extent of codon bias with compositional dynamics provides an insight into the prediction of the level of gene expression and genome characterization.

Advances in sequencing technology have provided an abundance of genomic data from different organisms. The study of CUB is gaining rehabilitated attention with the advent of whole genome sequencing of numerous organisms. Within a genome some genes are conserved and provide critical support to the organism, and these are termed as essential genes. The functions encoded by these genes are considered as a foundation of life itself. *Haemophilus influenzae* is a gram negative bacterium, highly adapted to its human host. The entire genome sequence of *Haemophilus influenzae* was completed and published in 1995. In the present work, the purpose was to study the CUB by analyzing the codon adaptation index (CAI), codon bias index (CBI), frequency of optimal codon (Fop), effective number of codons (ENC) and compositional dynamics for the essential genes of *Haemophilus influenzae*.

Materials and methods

Sequence data

For the present study there were selected genes that are essential for the growth or survival of *Haemophilus influenzae*. For CUB and compositional analyses were selected only those coding sequences (cds) having perfect initiator codon, terminator codon, devoid of any unknown bases (N) and are perfect multiple of three bases. The final data set consisted of twenty five (25) cds from

Haemophilus influenzae. Complete nucleotide coding sequence for each of the concerned gene satisfying the aforementioned criteria was retrieved from NCBI nucleotide database.

Models

ENC, CBI and Fop were used to quantify the variation in codon usage bias for the cds. ENC is generally used to measure the codon usage bias of a gene that is independent of the gene length and the number of amino acids (Wright, 1990). The ENC value ranges from 20-61. Fop is used to measure the codon usage bias in a gene (Zhou *et al*., 2005). The Fop value ranges from 0.36 for a gene in which codon usage pattern is uniform to 1 for a gene in which codon usage is highly biased (Zhou *et al*., 2005). Similar to Fop, CBI also measures the extent to which preferred codons are used in a gene (Bennetzen and Hall, 1982). CBI value is normalized between -1 and 1. Thus, CBI value of 1 means only preferred codons are used, zero means random choice and less than zero implies greater use of non preferred codons (Bennetzen and Hall, 1982).

CAI is used to estimate the level of gene expression of the cds selected in the present study. CAI measures the degree of bias towards the codons in highly expressed genes and thus assesses the effective selection which helps in shaping the codon usage pattern (Naya *et al*., 2001; Gupta *et al*., 2004). The value of CAI ranges from 0 to 1. For a gene in which all synonymous codons are used equally, the CAI would be 0 indicating no bias and if optimal codons are used, the value will be 1 for the strongest codon bias (Stenico *et al*., 1994).

GC content at three codon positions *i.e.* GC1, GC2, GC3 was calculated to quantify the relationship between codon usage variations. GC content at the three codon position is presumed to be a good indicator of base composition bias (Zhou *et al*., 2005).

Analysis tools

All the above mentioned parameters except CAI for the CUB and compositional analysis were carried out by using the online tool CodonW. The CAI value for each of these cds was computed by using the software acua available for non-commercial purposes.

Results

The coding sequence of 25 essential genes was retrieved and their nucleotide composition bias and codon usage bias were analyzed. The results of the compositional analysis with the accession numbers for each cds are given in Tab. 1. To find out the relationship between the extent of codon bias and nucleotide composition the values of A, T, G, C and GC were compared with the A3, T3, G3, C3 and GC3 values, respectively. Highly significant positive correlations were observed between GC3 and A ($r=0.926$, $p<0.01$), GC3 and T ($r=0.933$, $p<0.01$), GC3 and G ($r=0.973$, $p<0.01$), GC3 and C ($r=0.966$, $p<0.01$), GC3 and GC ($r=0.984$, $p<0.01$) contents. Simultaneously, GC1, GC2 and GC3 values were calculated for each gene to investigate the relationship between codon usage variation and compositional constraints. The GC3% of the cds was in the range between 13.7 to 21 with a mean of 16.86 and standard deviation of 1.78. Moreover, while comparing the GC content at first codon position (GC1) and second codon position (GC2) with that of the third codon position (GC3) (Fig. 1) a striking positive correlation ($r=0.968$, $p<0.01$) was observed, indicating that the patterns of base compositions are most likely the result of mutation pressure rather than that of natural selection, since at all codon positions its effects are present.

Tab. 1. Compositional analysis of *Haemophilus influenzae* genes with their accession numbers

Sl. No	Genes	Accession Numbers	Nucleotide contents										
			A	T	G	C	A3	T3	G3	C3	AT	GC	GC3
1.	Glyceraldehyde-3-phosphate dehydrogenase	*NP_438174.1*	308	296	218	198	95	65	144	35	604	416	179
2.	Metalloprotease	*NP_438177.1*	142	122	126	75	30	26	67	31	264	201	98
3.	Formate dehydrogenase accessory protein FdhE	*NP_438182.1*	313	263	160	173	81	69	92	60	576	333	152
4.	Ribosomal-protein-alanine N-acetyltransferase	*NP_438183.1*	140	146	95	60	40	41	42	23	286	155	65
5.	GTP-binding protein Era	*NP_438186.1*	297	261	199	152	95	45	107	55	558	351	162
6.	Ribonuclease III	*NP_438187.1*	215	184	154	131	57	41	74	55	399	285	129
7.	Signal peptidase I	*NP_438188.1*	301	340	242	167	82	85	120	62	641	409	182
8.	Uracil-DNA glycosylase	*NP_438191.1*	215	177	130	138	54	44	65	56	392	268	121
9.	Hypothetical protein HI0020	*NP_438193.1*	369	484	309	278	144	121	153	61	853	587	214
10.	Citrate lyase subunit alpha	*NP_438195.1*	431	452	332	288	131	90	195	84	883	620	279
11.	Citrate lyase subunit beta	*NP_438196.2*	249	245	203	161	68	53	114	50	494	364	164
12.	Lipoate-protein ligase B	*NP_438200.1*	196	204	130	109	54	43	64	51	400	239	115
13.	Lipoprotein	*NP_438203.1*	321	244	154	145	108	56	78	45	565	299	123
14.	Penicillin-binding protein 2	*NP_438205.1*	642	560	417	337	195	133	206	117	1202	754	323
15.	Hypothetical protein HI0035	*NP_438208.1*	411	555	412	278	145	115	197	94	966	690	291
16.	Rod shape-determining protein MreC	*NP_438211.1*	366	294	195	201	103	49	118	81	660	396	199
17.	Rod shape-determining protein MreD	*NP_438212.1*	123	195	83	88	38	57	35	32	318	171	67
18.	Hypothetical protein HI0040	*NP_438213.1*	260	270	150	97	70	68	80	40	530	247	120
19.	Exonuclease III complete cds	*NP_438214.1*	261	238	173	132	69	57	87	54	499	305	141
20.	Pseudouridine synthase-like protein	*NP_438215.1*	228	209	133	105	71	49	59	45	437	238	104
21.	Hypothetical protein HI0043	*NP_438216.1*	349	336	224	156	98	80	109	67	685	380	176
22.	Hypothetical protein HI0045	*NP_438218.1*	193	160	128	73	62	33	66	22	353	201	88
23.	D-mannonate oxidoreductase	*NP_438221.1*	289	274	163	132	96	62	96	31	563	295	127
24.	2-dehydro-3-deoxygluconokinase	*NP_438222.1*	304	307	186	148	86	74	94	60	611	334	154
25.	Hypothetical protein HI0056	*NP_438229.1*	199	246	158	111	77	48	74	38	445	269	112

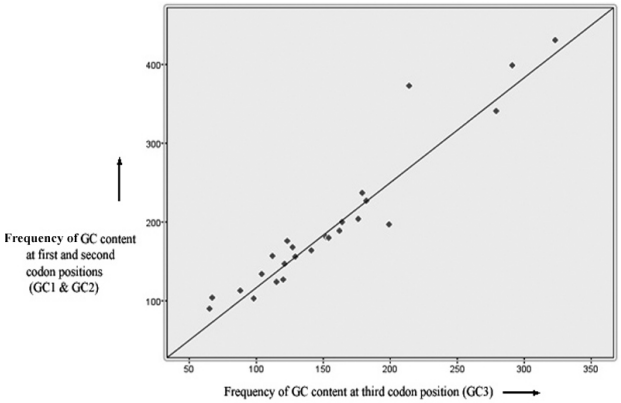


Fig. 1. Correlation between GC content at first and second codon positions (GC1 & GC2) with that at third codon position (GC3). GC12 is the average of GC content at first and second codon positions

Wright (1990) suggested that a plot of ENC against GC3s could be effectively used to explore the codon usage variation among the genes (Wright, 1990). We computed the ENC values and compared with the frequency of GC3s (Fig. 2). A wide variation of CUB among the genes was observed indicating that the distribution of GC3 is not the only determinant; apart from GC3s, compositional constraints and other trends might influence the overall codon usage variation among the genes of *Haemophilus influenzae*. Moreover, we compared the ENC value and GC3 value with gene length to measure the relationship between codon usage bias and gene length. We have plotted ENC value against gene length and observed that the shorter genes had a much wider variation in ENC values and vice versa for longer genes (Fig. 3). The analysis showed a significant negative correlation (Pearson one tail, r= -0.368, $p<0.05$) between ENC and gene length, but significant positive correlation (Pearson r=0.976, $p<0.01$) between GC3 and gene length, respectively Figs. 4(a), (b).

Gene expression level was measured using CAI values (Gupta *et al*., 2004; Behura and Severson, 2012), which varied from 0.645 to 0.764 with a mean of 0.70 and standard deviation of 0.037. Since the essential genes are required for the growth and/or survival of the organism, their rate of expression/transcription needs to be finely regulated. From the CAI analysis it was evident that all the 25 cds encompasses the codons in such a way that they can express at high rate. This further confirms that the genes selected for the analysis are essential for *Haemophilus influenzae*. Furthermore, significant negative correlation was also observed between ENC and CAI (r= -0.72, $p<0.01$). Comparison of the frequency of optimal codons (Fop) with CAI value as an indicator of gene expression revealed that CAI *i.e.* the level of gene expression increases with the rise of Fop value (Fig. 5).

Moreover, significant positive correlation was observed between CBI and CAI (Pearson r=0.853). These results altogether suggest that the codon usage pattern determines the level of gene expression in *Haemophilus influenzae*.

Discussion

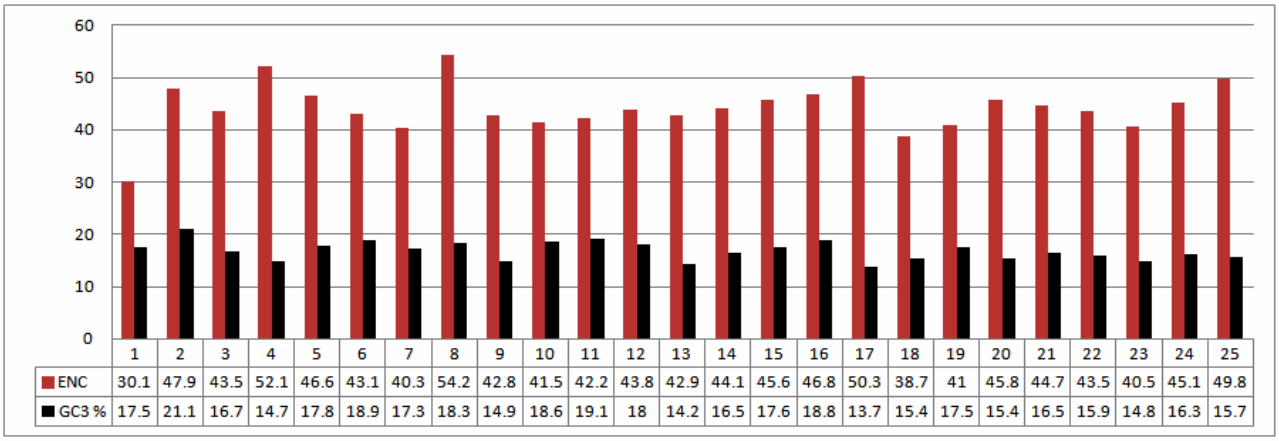
The present study was taken up to analyze several widely used parameters of codon usage bias namely CAI, CBI, Fop and ENC along with the base composition of the coding sequences of some essential genes in *Haemophilus influenzae*. The accurate coding sequences were retrieved using a program in perl, developed by the researchers involved in the current study. After preliminary analysis of base composition it was found that the cds of *Haemophilus influenzae* are rich in AT.

A complex correlation was observed while investigating the relationship between different nucleotide constraints. T3 had a significant positive correlation with A (r=0.831, $p<0.01$), T (r=0.956, $p<0.01$), G (r=0.884, $p<0.01$), C (r=0.863, $p<0.01$) and GC (r=0.888, $p<0.01$). Similarly, C3 had also significant positive correlation with C (r=0.862, $p<0.01$), T (r=0.816, $p<0.01$), A (r=0.871, $p<0.01$), G (r=0.841, $p<0.01$) and GC (r=0.863, $p<0.01$). A3 had significant positive correlation with A (r=0.957, $p<0.01$), T (r=0.931, $p<0.01$), G (r=0.906, $p<0.01$), C (r=0.920, $p<0.01$) and GC (r=0.926, $p<0.01$). Similarly, G3 had also significant positive correlation with G (r=0.971, $p<0.01$), C (r=0.951, $p<0.01$), T (r=0.927, $p<0.01$), A (r=0.890, $p<0.01$) and GC (r=0.977, $p<0.01$).

Moreover, the indices GC1, GC2, GC3 and GC12 (average of GC1 and GC2) were computed for each gene to establish the relationship among three codon positions. Our results showed that the coding sequences of *Haemophilus influenzae* have a wide range of GC3 and this difference usually influences the neutral mutation bias, leading to different codon choice in each gene (Liu *et al*., 2012). Concurrently, a significant positive correlation of GC12 with GC3 (r=0.968, $P<0.01$) and GC1 with GC3 (r=0.955, $p<0.01$) were observed; this may be due to the intragenomic GC mutational bias affecting the GC contents at all codon positions.

Several studies revealed that both natural selection and mutation pressure account for codon usage variation among different organisms. If synonymous codon usage bias is affected only by mutational pressure, then the frequency of nucleotides A and T should be equal to that of C and G at the synonymous third codon position (Zhang *et al*., 2013). However, the current study revealed wide variations in the nucleotide A and T with C and G base compositions, signifying that other factors, such as natural selection, might also be the determining factor in shaping the synonymous codon usage pattern. In general, there is an inverse relationship between ENC and gene expression *i.e.* a lower ENC value indicates a higher codon usage preference and higher gene expression and vice versa (Wright, 1990). Our results revealed that the overall codon usage bias among different genes of *Haemophilus influenzae* is low, slightly biased and mainly affected by base composition.

The CAI value was found in the range 0.64 to 0.76 for the cds, further indicating low codon usage bias and relatively high gene expression level, similar to the ENC analysis. More interestingly, a significant correlation was observed between ENC and CAI (r=-0.72) which indicates that the essential genes have a strong preference for a subset of codons, as shown by the positive correlation between optimal codons and tRNA abundance (Ikemura, 1981). Selection for translational accuracy is predicted to have a positive correlation between codon bias and gene length (Eyre-Walker, 1996). From the plot drawn with gene length against ENC (Fig. 3), it is



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
ENC	30.1	47.9	43.5	52.1	46.6	43.1	40.3	54.2	42.8	41.5	42.2	43.8	42.9	44.1	45.6	46.8	50.3	38.7	41	45.8	44.7	43.5	40.5	45.1	49.8
GC3 %	17.5	21.1	16.7	14.7	17.8	18.9	17.3	18.3	14.9	18.6	19.1	18	14.2	16.5	17.6	18.8	13.7	15.4	17.5	15.4	16.5	15.9	14.8	16.3	15.7

Fig. 2. Frequency of GC3s and the distribution of ENC values of 25 selected coding sequences

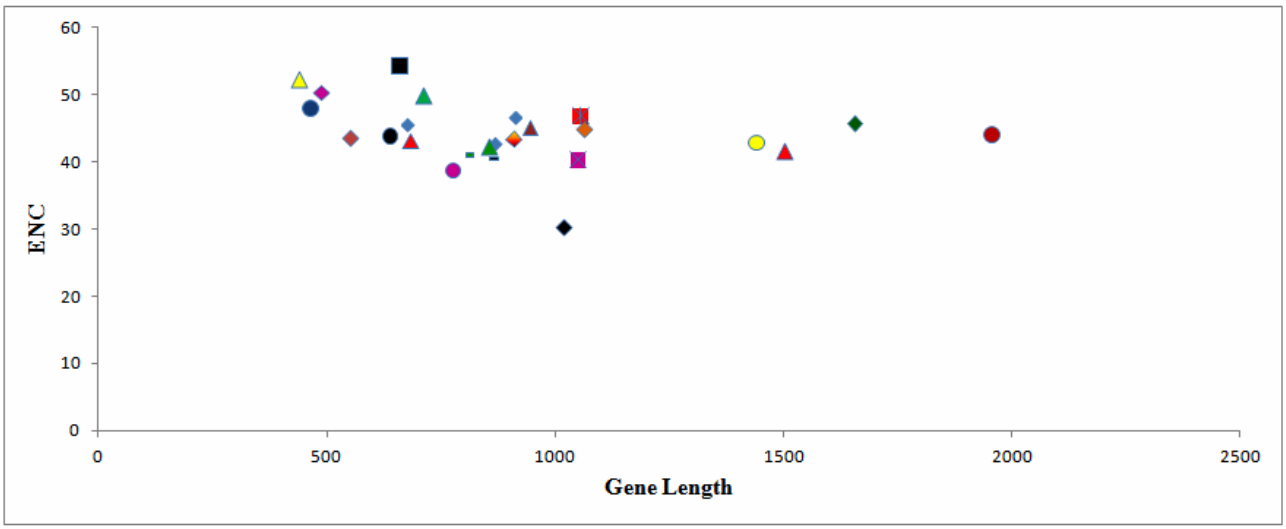


Fig. 3. Plot of ENC versus gene length for the selected cds of *Haemophilus influenzae*

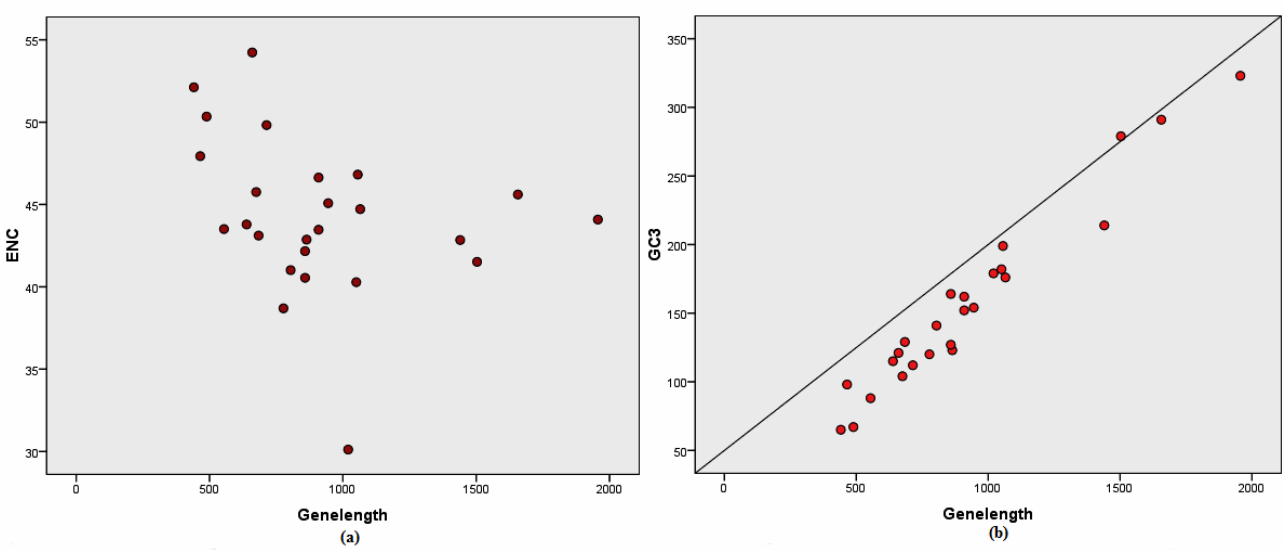


Fig. 4. Correlation between (a) ENC values and gene length (bp); (b) GC3 values and gene length (bp)

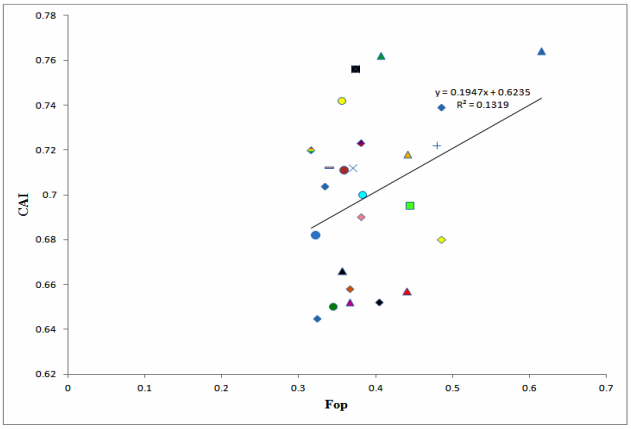


Fig. 5. Relation between the frequency of optimal codon usage and gene expressivity

understood that shorter genes have a much wider variance in ENC values, but vice versa for longer genes. Furthermore, a significant negative correlation between ENC and gene length revealed that gene length is one of the important factors which influence codon usage in the cds of *Haemophilus influenzae*. Lower ENC values in longer genes may be due to the direct effect of translation time on fitness or the extra energy cost of proofreading associated with longer translation time (Hassan *et al*., 2009; Liu *et al*., 2003).

Conclusion

In brief, our analysis showed that overall codon usage bias in the essential genes of *Haemophilus influenzae* is slightly biased and mutation pressure played a major role to shape the codon usage pattern in these genes. In addition, the contributions of other factors such as compositional bias, translational forces and natural selection are also evident in shaping the codon usage pattern of *Haemophilus influenzae*. From the present analysis it can be concluded that the essential genes in *Haemophilus influenzae* preferred AT to GC bases (compositional analysis) within their coding sequences and this preference might affect gene expression as indicated by the relatively high CAI values of the cds.

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