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RESEARCH ARTICLE

Evolution of tryptophan biosynthetic pathway in microbial genomes: a comparative genetic study

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Abstract Biosynthetic pathway evolution needs to consider the evolution of a group of genes that code for enzymes catalysing the multiple chemical reaction steps leading to the final end product. Tryptophan biosynthetic pathway has five chemical reaction steps that are highly conserved in diverse microbial genomes, though the genes of the pathway enzymes show considerable variations in arrangements, operon structure (gene fusion and splitting) and regulation. We use a combined bioinformatic and statistical analyses approach to address the question if the pathway genes from different microbial genomes, belonging to a wide range of groups, show similar evolutionary relationships within and between them. Our analyses involved detailed study of gene organization (fusion/splitting events), base composition, relative synonymous codon usage pattern of the genes, gene expressivity, amino acid usage, etc. to assess inter- and intra-genic variations, between and within the pathway genes, in diverse group of microorganisms. We describe these genetic and genomic variations in the tryptophan pathway genes in different microorganisms to show the similarities across organisms, and compare the same genes across different organisms to find the possible variability arising possibly due to horizontal gene transfers. Such studies form the basis for moving from single gene evolution to pathway evolutionary studies that are important steps towards understanding the systems biology of intracellular pathways.

Keywords Pathway evolution · Tryptophan biosynthetic pathway · Relative synonymous codon usage (RSCU) · Relative amino acid usage (RAAU) · Codon adaptation index (CAI) · Principal component analysis (PCA) · Cluster analysis

Introduction

Sequencing large number of prokaryotic genomes from a variety of phyla and classes has offered new opportunities to study prokaryotic evolution. There are several methods to study molecular evolution of genomes through gene transfers. The commonly used one is through searching for sequence similarity using phylogenetic models (Felsenstein 2003), but a number of other approaches include identification of codon usage patterns, base composition (GC content) analysis, and nucleotide-pattern properties within genomes that differ from the genomic norm, as these are likely to represent acquired sequences (Moszer et al. 1999; Mrazek and Karlin 1999; Nakamura et al. 2004). In spite of many studies across genomes and for specific genes, a comprehensive understanding of how one can study such properties in groups of genes coding for the enzymes in the same metabolic pathway in different genomes, remain poorly understood.

Pathway evolution has been studied from the "origin of life" context, and two hypotheses have been put forward to explain the evolution of the sequence of steps in a pathway

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(Rison and Thornton 2002). In this study we attempt to study pathway evolution from a different perspective. Intracellular biosynthetic pathways consist of multiple reaction steps catalysed by enzymes, whose genes generally form a group (operon) in microorganisms. All genes of the enzymes involved in the pathway need to be concurrently functional for the end product to be available, so that the entire pathway can operate in the organisms for generations (Hao and Golding 2006). Thus this group of genes evolves under similar constraints determined by the functionality of the complete pathway, and hence they are expected to co-evolve. Hence, pathway evolution needs to consider the evolution of a group of genes, and this may entail all the genes in the same pathway to be under similar selection pressure. It is only recently that similar questions are being addressed as to how natural selection shapes the evolution of the group of genes for enzymes participating in specific biochemical pathways (Flowers et al. 2007; Invergo et al. 2013).

In this work, we have addressed if the systems-level constraints, operating at the whole pathway level (i.e., to produce the end product), influence the genetic and genomic features of the genes for the constituent enzymes in a correlated manner across different genomes. To achieve this we have studied the codon usage patterns, base composition and usage in third codon position, amino acid usage, and nucleotide-pattern properties of the constituent enzymes in a metabolic pathway. From the composition-based features of these genes, we examine the possibilities of gene transfer events within and between the genomes of different microorganism. It is known that organisms exhibit different preferred usage of the synonymous codons for the same amino acid, and the relative frequencies of different synonymous codons vary both within and between organisms (Grantham et al. 1980, 1981; Gouy and Gautier 1982; Lynn et al. 2002). Since codon usage varies from genome to genome, comparing the codon usage for all genes in a genome can help identify unusual nucleotide or codon usage in a gene and infer about lateral gene transfer (LGT) from another species (Kurland et al. 2003; Eisen 2000; Garcia-Vallve et al. 2003; Grocock and Sharp 2002; Gupta and Ghosh 2001; Kunin and Ouzounis 2003; Watt and Dean 2000). Other studies have indicated that diverse patterns of codon usage may arise from compositional constraints of the genomes as observed in the case of extremely GC- or AT-rich organisms (Karlin and Mrazek 1996; Ghosh et al. 2000).

We have considered the metabolic pathway for the biosynthesis of the amino acid Tryptophan as a case to study the above-mentioned features in a selected set of genomes spanning different phyla of bacteria and archaea. The choice of tryptophan biosynthetic pathway for our study is based on several issues. Tryptophan being the most energetically expensive amino acid to be synthesized in a cell (Bentley 1990; Akashi and Gojobori 2002), tryptophan biosynthesis is expected to be tightly controlled (Yanofsky et al. 1981, 2001). Though evolutionary studies on this pathway have been of interest for quite some time (Crawford 1975, 1989), the accumulation of genome sequences from a diverse group of organisms has enabled thorough and elaborate analysis of the evolution of the pathway in terms of the variation in gene arrangements, operon structure and regulation in a large number of microorganisms. A seminal review and a series of in-depth analysis of the trp operons in diverse groups of bacteria, archaea, and fungi genome sequences (Xie et al. 2001, 2002, 2003a, b, 2004) have demonstrated that the trp operon has been "organizationally reshuffled, invaded by insertion of apparently unrelated genes, disrupted by either partial or complete dispersal of genes to extra-operonic locations, or complicated by the seemingly unnecessary presence of additional operon-gene copies located outside of the operon". These comprehensive studies analyzed the comparative organisation of the seven structural genes of the enzymes of the pathway, highlight the role of regulation, and elucidate some key organisms, which represent major evolutionary events in gene organization, and possible routes of lateral gene transfers. Needless to say, the tryptophan pathway gene clusters, though provide strong clues to vertical descent, has enough examples of mosaicism due to gene transfers by LGT or other events (Xie et al. 2003b, 2004; see Merino et al. 2008 for an excellent summary).

Genes in an operon typically have closely related functions, and consequently there is a strong selective pressure for the operon structure to be conserved between genomes (Lawrence and Roth 1996). In this study we have used a comparative genomic approach, complemented by statistical analysis, to study the evolution of the tryptophan biosynthetic pathway genes by analyzing their codon usage pattern along with the gene expressivity data in 15 microbes from different lineages (Table 1). The choice of organisms is based on several considerations—events of gene fusion/splitting in operon organization (Xie et al. 2003a, b), varying GC content of the genomes, variable number of genes in their trp operon, and organisms from taxonomically different lineages such as proteobacteria and archaea. Figure 1 gives the evolutionary tree (based on the 16 s rRNA genes for these organisms) showing their phylogenetic groupings. A comparison of Table 1 and Fig. 1 shows that organisms from same phylogenetic groups can have different base compositions and gene organisations.

The tryptophan branch of the aromatic amino acid group (Tyrosine, Phenylalanine and Tryptophan) proceeds from Chorismic acid, as shown in Fig. 2. It consists of a series of five chemical reactions (Crawford 1975) that involves five enzymes, encoded by 5–7 genes (trpEGDFCBA) in different

Table 1 Trp operon gene organization and whole genome GC content of 15 microbes from two lineages (12 Bacteria and 3 Archaea) and eight phyla. (**trpF* in *Mtu* and *Sco* is a bifunctional (*hisA*-*trpF*) gene (Barona-Gómez and Hodgson 2003)

Lineage	Organisms	Number of genes	% GC content
Bacteria			
γ—proteobacteria	*Escherichia coli* (K-12 MG1655) (Eco)	5 (trpE-GD-FC-B-A)	50.8
	Salmonella typhi (CT18) (*Sty*)	5 (trpE-GD-FC-B-A)	52.2
	Pseudomonas aeruginosa (PA01) (*Pae*)	7 (trpE-G-D-F-C-B-A)	66.6
	Coxiella burnetti (RSA 493) (Cbu)	5 (trpE-G-D-BF-A)	42.6
α—proteobacteria	*Brucella melitensis (16 M)* (*Bme*)	6 (trpEG-D-F-C-B-A)	57.2
	Mesorhizobium loti (mlr2744) (*Mlo*)	6 (trpEG-D-F-C-B-A)	62.5
ε—proteobacteria	*Helicobacter pylori (26695)(Hpy)*	6 (trpE-G-D-FC-B-A)	38.9
Thermotogae	*Thermotoga maritima* (*Tma*)	6 (trpE-GD-F-C-B-A)	46.2
Actinobacteria	*Mycobacterium tuberculosis (H37Rv)* (*Mtu*)*	5 (trpE-D-F-C-B-A)	65.6
	Streptomyces coelicolor (*Sco*)*	5 (trpE-G-D-F-C-B-A)	72.2
Firmicutes	*Bacillus subtilis (00750)* (*Bsu*)	7 (trpE-G-D-F-C-B-A)	43.5
	Streptococcus pneumoniae (TIGR4) (*Spn*)	7 (trpE-G-D-F-C-B-A)	39.7
Archaea			
Euryarchaeota	*Archaeoglobus fulgidus* (*Afu*)	6 (trpE-G-C-F-B-A-D)	48.6
	Methanobacterium thermoautotrophicum (*Mth*)	7 (trpE-G-D-FC-B-A)	49.5
Crenarchaeota	*Sulfolobus solfataricus* (*Sso*)	7 (trpE-G-D-F-C-B-A)	35.8

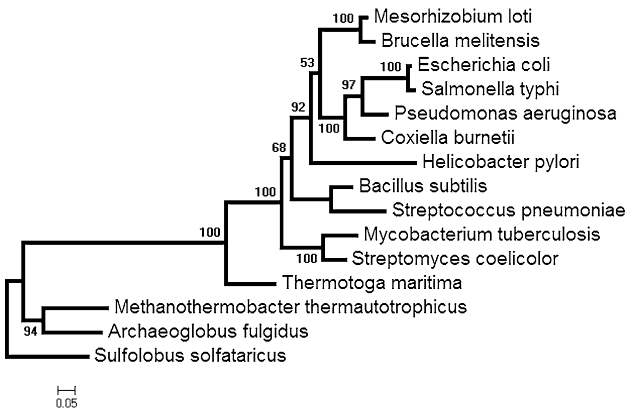


Fig. 1 Phylogenetic tree of 16s rRNA of the organisms under study

microbes. The genes are usually arranged in a single cluster (trp operon), and codes for different chains for different enzymes as shown by the dashed lines. The first two genes *trpE* and *trpG* encode the two subunits of the enzyme Anthranilate Synthase (AS), which catalyses the chemical reaction step from the substrate Chorismate (1) to Anthranilate (2). Gene *trpG* is fused with *trpD* in some bacteria (such as, *E. coli.*), but except for two, are always split in Archaea Gene *trpD* codes for Anthranilate Phosphoglycerol Transferase (PRT), which catalyses the reaction step (2) to (3). Again, *trpF* and *trpC*, coding for *N*-(5′-Phospho-Ribosyl)-Anthranilate Isomerase (PRAI) and Indole-3-Glycerol Phosphate Synthase (IGPS), catalyze the reactions from steps (3) to (4) and steps (4) to (5) respectively, are found fused in some bacteria, but always are separate in Archaea. The genes *trpB* and *trpA* are never found fused in prokaryotes (Xie et al. 2003a, b), and both encode for the β and α subunits of the enzyme Tryptophan Synthase (TS), which catalyzes the reaction steps (5) to (6) finally making the end product amino acid L-Tryptophan.

The chemical reactions of the pathway steps are highly conserved in a diverse group of organisms, but the genes corresponding to the five enzymes have considerable variation in their arrangements, operon structure and regulation in different bacteria, which continue to be an active area of study (Crawford 1975; Merino et al. 2008). The gene fusion/splitting events in the Trp operon organization are present in all lineages. Of the seven genes, the commonly observed fusions are G*D (e.g. *Eco*, *Sty*, and *Tma*), C*F (most of γ-proteobacteria, except *Pae*), even though D*C and E*G also exist. In the group of organisms chosen by us represents lineages that contain all the members of the trp gene “module”, but organized in different combinations. We have presented in this study a comprehensive analysis of the intra- and inter-genic variations in the base composition, amino acid usage, codon adaptation, gene expressivity, and multivariate analysis of their synonymous codon usage pattern, both within and between organisms of very different lineages. Given the fact that this “module” of genes underlies a specific function, and the organisms chosen lie at different branches of the microbial species tree, our study can shed light on the extent of gene-specific and species-specific variations in the same pathway genes, in the background of a significant vertical transmission of information though evolution. Examining the differences in these composition-based features may be indicative of differences in their evolutionary origin (vertical or lateral

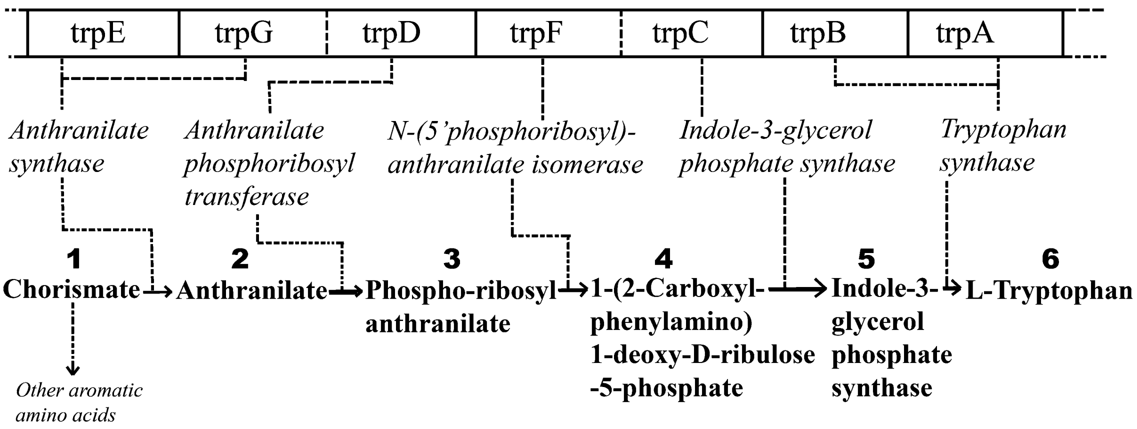

trpE
trpG
trpD
trpF
trpC
trpB
trpA
Anthranilate synthase
Anthranilate phosphoribosyl transferase
N-(5'phosphoribosyl)-anthranilate isomerase
Indole-3-glycerol phosphate synthase
Tryptophan synthase
1 Chorismate
2 Anthranilate
3 Phospho-ribosyl anthranilate
4 1-(2-Carboxyl-phenylamino) 1-deoxy-D-ribulose -5-phosphate
5 Indole-3-glycerol phosphate synthase
6 L-Tryptophan
Other aromatic amino acids

Fig. 2 Schematic diagram of substrates, genes, and gene-products (enzymes) catalyzing the reactions in Tryptophan biosynthetic pathway in *Escherichia coli* (*Eco*)

gene transfers) within the same module of genes underlying the same function.

Materials and methods

Source of sequence data

The nucleotide sequences for the Tryptophan biosynthetic pathway genes for 15 organisms (Table 1) from different groups of bacteria and archaea were retrieved from the KEGG pathway database Ortholog table (http://www.genome.jp/kegg). The nomenclature for the names of the genes is taken from KEGG (Kanehisa et al. 2007). The whole genome sequences, highly expressed genes and the 16S rRNA sequences were obtained from NCBI entrez genome project (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The whole genome sequences were downloaded by selecting the corresponding organism's REFSEQ displayed in cDNA/FASTA format. The highly expressed gene-set was made by taking only ribosomal genes separately for each organism from Tools/Protein table. (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

Phylogenetic analysis

The sequences for the 16 s rRNA tree and *trpA* were aligned using MUSCLE (Edgar 2004) and the phylogenetic tree was obtained using Phylip (Felsenstein 1989) using the kitsch program that implements the Fitch-Margoliash and Least Squares method. The trees were drawn using MEGA5 (Tamura et al. 2011).

Base composition analysis

The base composition of DNA refers to the content of the four nucleotides, Guanine (G), Cytosine (C), Adenine (A), and Thymine (T), in the sequence. Thus, GC content of a fragment of DNA refers to the content of the single nucleotides, G+C, and AT content refers to A+T content in that fragment. Organisms in Table 1 were chosen with a large range of variation of GC content—78–35 %. The frequency of the bases at the synonymous third position of the codon is indicated by A3 for Adenine, T3 for Thiamine and GC3 for Guanine+Cytosine. The average base composition of all the genes of enzymes involved in the tryptophan biosynthetic pathway is referred to as "pathway base content". Mean whole-genome GC3 refers to the average base composition in the third position of the codons of all the genes in the genome.

Analysis of gene expressivities

Gene expressivities were measured using *Codon Adaptation Index*, CAI (Sharp and Li 1986; Carbone et al. 2003; Jansen et al. 2003).

$$\mathrm{CAI} = \exp\left[\frac{1}{\mathrm{L}}\sum_{\mathrm{i}=1}^{18}\sum_{\mathrm{j}=1}^{\mathrm{n_i}}\mathrm{X_{ij}}\ln \mathrm{w_{ij}}\right]$$

where, L is the sum total of the number of occurrences of all the codons for 18 amino acids present in the query gene and w_{ij} (adaptiveness) is the normalized RSCU value of the jth codon of ith amino acid of reference set of highly expressed genes.

This measure ranges between 0 and 1, where a value greater than 0.5 indicates a greater level of codon bias, and predicts a higher protein level. The CAI was calculated using CodonW.

Relative amino acid usage (RAAU)

RAAU represents the fractional content of a particular amino acid among all the 20 amino acid residues within a gene product, calculated using CodonW. The RAAU of all the pathway genes were compared individually with that of their whole genomes for each organism to find whether these pathway genes exhibit any difference in the pattern of amino acid usage. Amino acid usage are categorized as follows (Chanda et al. 2005): more than 10 %—Most Abundant; 6–10 %—Abundant; 4–6 %—Intermediate; 2–4 %—Rare; and less than 2 %—Very Rare. The RAAU values of *trpG* for *Mtu* was not plotted because the gene is listed as a putative gene in NCBI.

Relative synonymous codon usage (RSCU)

RSCU is defined as the ratio of the observed frequency of a codon to the expected frequency, if all the synonymous codons for those amino acids are used equally. It is used to study the overall codon usage variation among the genes. It is evaluated from the following mathematical expression (Sharp and Li 1987)

$$\mathrm{RSCU}_{\mathrm{ij}} = \frac{\mathrm{X}_{\mathrm{ij}}}{\left(\frac{1}{\mathrm{n_i}}\right)\sum_{\mathrm{i}=1}^{\mathrm{n_i}} \mathrm{X}_{\mathrm{ij}}}$$

where, X_{ij} is the number of occurrences of the jth codon for the ith amino acid, and n_{i} is the number (from 1 to 6) of alternative codons for the ith amino acid. RSCU values were calculated for each gene in the pathway for the 15 organisms using CodonW (Peden 1999; http://codonw.sourceforge.net/) for 59 codons excluding 5 codons viz. AUG (methionine), UGG (tryptophan), and the three termination codons, as they give no bias.

To avoid biases due to gene length, amino acid usage, and codon degeneracy, codon usage data was normalized using the following formula (Suzuki et al. 2005).

$$\mathrm{nor.RSCU}\left(\mathrm{w}_{\mathrm{ij}}\right) = \mathrm{RSCU}_{\mathrm{ij}}/\mathrm{RSCU}_{\mathrm{i(max)}}$$

Correspondence analysis (COA)

COA is a method to factor categorical variables and display them in space so that association in two or more dimensions can be visualized. COA on RSCU (Benzecri 1992; Greenacre 1984; Das et al. 2005) of highly expressed genes (ribosomal genes) and trp genes were done to check the trend of codon usage preference between the two.

Multivariate analysis

Multivariate Analysis was done using *Principal Component Analysis* (PCA) and *Cluster Analysis* (CA) techniques.

Cluster Analysis is a method of classification of objects into groups, which reveal the relationships existing between the groups. The CA Plots are easier to interpret (Sharp et al. 1986). *Cluster Analysis* was done using MATLAB 2012b on the normalized RSCU for 59 codons of each gene. The two key steps considered within cluster analysis were the measurement of pair wise (Euclidean) distances between objects, and grouping the objects based upon the resultant linkage (unweighted pair group average) distances.

The purpose of *Principal Component Analysis* (PCA) is used to classify datasets. *Principal Component Analysis* was carried out on the normalized RSCU data of 59 codons, with 59 variables corresponding to the 59 degenerate codons (excluding the codons for Met, Trp and the stop codons), with 15 observations corresponding to the normalized codon usage values for the 15 organisms under study for each gene of the pathway. PCA was done using MATLAB 2012b. The genes C-F and G-D were manually concatenated and RSCU were calculated in cases where they were split in order to avoid the difference in number of genes in the pathway. In all the genes the first two PCs accounted for 50–60 % variance and the first three PCs accounted for 60–70 % variance in the data. Details of the analysis is given in Supplementary material S2.

Results

Analysis of base content at third position

Diverse patterns of codon usage may arise from compositional constraints of the genomes depending on if they are from GC- or AT-rich organisms (Karlin and Mrazek 1996). In amino acids with more than two fold codon degeneracy, it is the third position that decides the translational efficiency, and the total codon usage drive of an organism, as the third position bias positively correlates with gene expression (Carlini et al. 2001). The frequency of Adenine (A3), Thiamine (T3), Guanine+Cytosine (GC3) content for each pathway gene for all 15 organisms from different bacterial lineages are shown in Fig. 3. The organisms *Afu, Sty, Bme, Mtu, Mlo, Pae* and *Sco*, which have a GC content ranging from 49 to 72 % show comparatively higher GC3 bias (62–97 %) in the pathway genes. Almost equal usage (44–58 %) is seen in *Bsu, Hpy, Tma, Mth* and *Eco,* and *Cbu, Spn* and *Sso* exhibit higher AT3 bias.

Figure 4a shows a comparative study of each organism's whole genome GC contents, and the mean GC and mean GC3 contents of the Trp pathway genes. Even though the average GC content of the pathway and whole genome is similar for all the organisms, significant variations exist in the GC3 preference of pathway genes among the organisms. The plot for mean whole genome GC3 and GC3 contents of the *Trp* pathway genes in different organisms are shown in Fig. 4b. It clearly shows that *Sty, Afu, Spn,* and *Sco* are GC3 skewed (>5 % difference), whereas *Sso, Cbu* and *Mtu* shows higher GC3 in the whole genome when compared to their mean pathway GC3 content. Thus, Figs. 3 and 4 indicate that, even

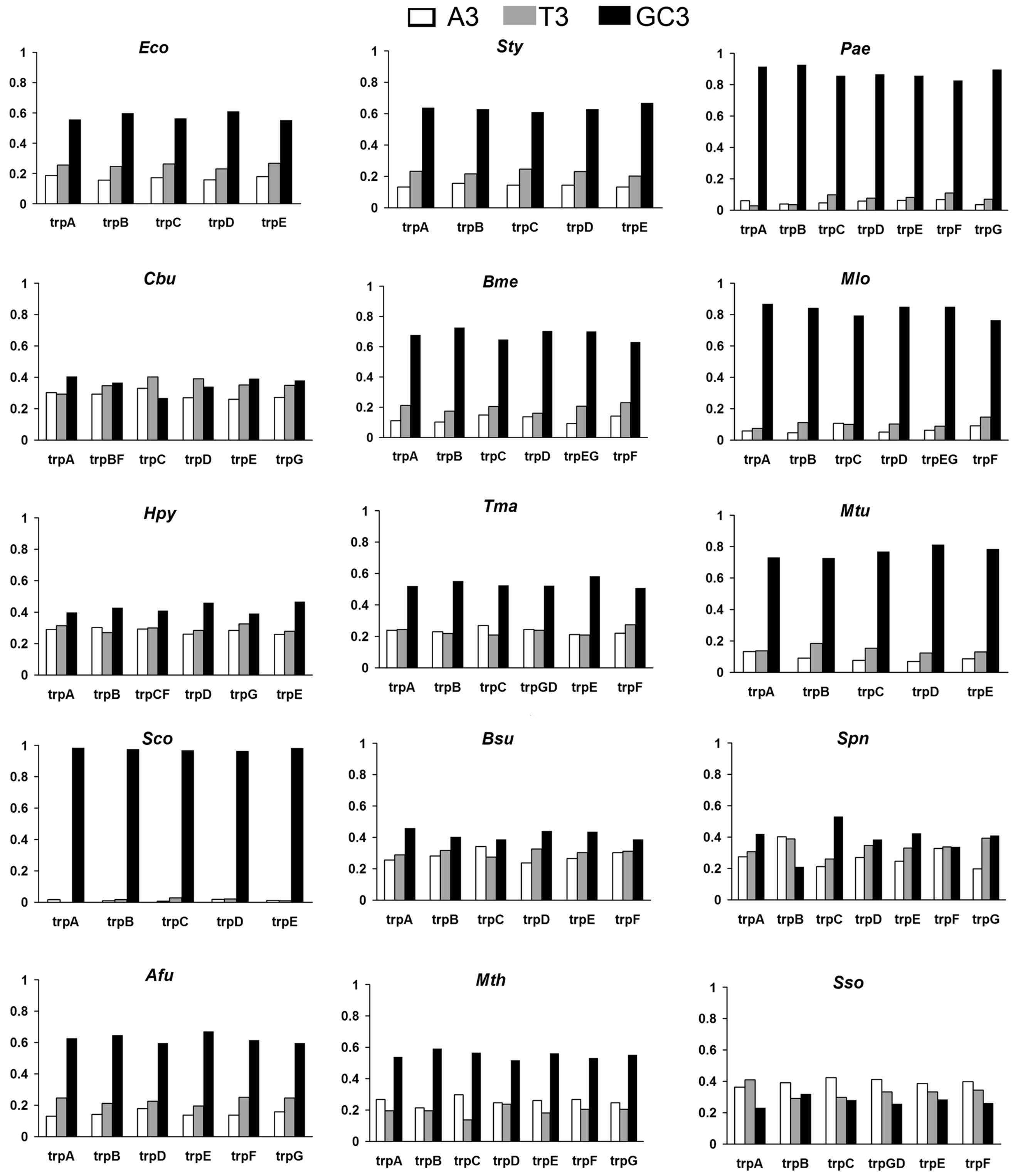


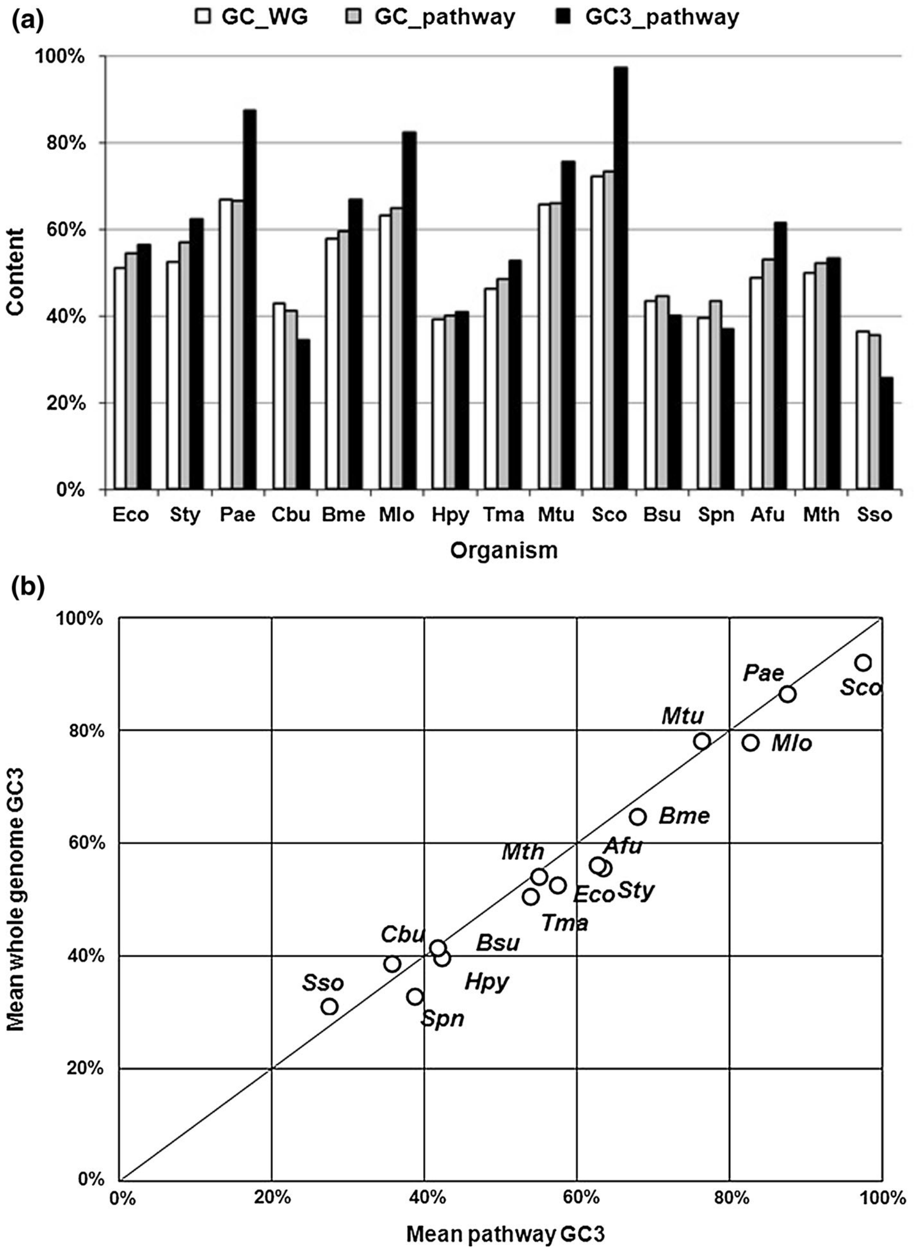
Fig. 3 Third position-specific bias in codons of *trp* genes in different organisms

though the average base composition of the Trp pathway genes are in accordance to their whole genome GC content, the GC3 usage varies among genes and show considerable skew in some organisms across phyla.

Gene expressivity analysis

Every organism has a preferred set of codons, for each amino acid, that occur most frequently in genes that are

Fig. 4 GC content analyses: **a** GC content of the whole genome, and mean GC and GC3 content of Trp pathway genes, **b** Mean GC3 content of whole genome and mean GC3 content of Trp pathway genes, in all organisms



translated at high abundance (e.g., the ribosomal proteins, or translation elongation factors) (Gouy and Gautier 1982). The patterns in the Codon Adaptation Index (CAI) are a useful measure to understand gene expressivity. In Table 2, we compare the CAI values of the Trp pathway genes to their ‘preferred’ codons—in this case, in the ribosomal proteins of each organism (Sharp and Li 1986).

It is clear from Table 2 that, with respect to the reference set of highly expressed ribosomal genes (HEGs), all the pathway genes show high codon adaptation (>0.5) in the three archaea (*Afu, Mth* and *Sso)* and seven bacterial species (*Mlo, Mtu, Sco*, *Hpy, Cbu,* and *Tma*). The three bacteria, *Eco, Bsu, Sty* and *Spn*, show moderate to low expressivity (0.3 < CAI < 0.5). All pathway genes in *Sty* have moderate but highly uniform CAI values.

In contrast, the two bacteria *(Pae* and *Bme)* show gene specific variability in CAI, most of the genes have higher expressivity except for *trpF*, which shows moderate expressivity.

The Correspondence Analysis (COA) plots (Supplementary material S1) are also in accordance with CAI analysis, where the Trp pathway genes in all archaea and few bacteria (*Sco*, *Tma*, *Cbu*) strongly cluster with the HEGs. These also show higher CAI (bold values in

Table 2 CAI values of Trp pathway genes with respect to highly expressed genes in different organisms

Org	Anthranilate synthase		Anthranilate phosphoribosyl transferase (TrpD)	Indole-3-glycerol phosphate synthase (TrpC)	Anthranilate isomerase (TrpF)	Tryptophan synthase	
	Comp I (TrpE)	Comp II (TrpG)				(TrpB)	(TrpA)
Eco	0.38	0.38	0.37	0.33	0.35	0.43	0.36
Sty	0.37	0.34	0.36	0.35	0.32	0.39	0.35
Pae	**0.62**	**0.65**	**0.6**	**0.65**	*0.49*	**0.77**	**0.69**
Cbu	**0.8**	**0.72**	**0.78**	**0.79**	**0.75**	**0.79**	**0.76**
Bme	**0.58**	**0.64**	**0.62**	**0.57**	*0.47*	**0.67**	**0.63**
Mlo	**0.69**	**0.64**	**0.6**	**0.54**	**0.51**	**0.69**	**0.7**
Hpy	**0.69**	**0.74**	**0.71**	**0.65**	**0.71**	**0.7**	**0.7**
Tma	**0.76**	**0.72**	**0.72**	**0.75**	**0.73**	**0.72**	**0.75**
Mtu	**0.61**	–	**0.58**	**0.59**	**0.6**	**0.55**	*0.48*
Sco	**0.72**	**0.68**	**0.66**	**0.68**	**0.75**	**0.76**	**0.75**
Bsu	0.38	0.38	0.37	0.4	0.41	0.41	0.37
Spn	0.43	0.48	**0.5**	0.38	0.41	**0.53**	0.49
Afu	**0.76**	**0.67**	**0.72**	**0.72**	**0.72**	**0.74**	**0.71**
Mth	**0.69**	**0.7**	**0.67**	**0.66**	**0.61**	**0.71**	**0.65**
Sso	**0.68**	**0.66**	**0.67**	**0.64**	**0.67**	**0.68**	**0.7**

Values in bold and regular indicate higher and, moderate expressivity, respectively. The italic values indicate the gene in the operon with *comparatively* low expression

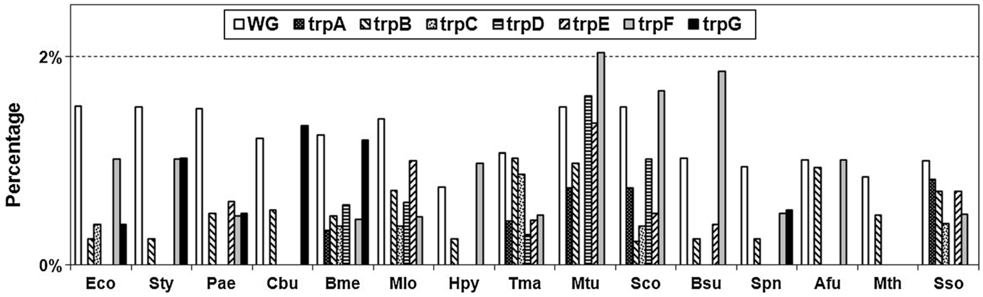


Fig. 5 Usage of Tryptophan by Trp pathway genes in different organisms. *WG* whole genome

Table 2). The genes *trpC, trpF* and *trpG* are the three genes that remain outside the major cluster in several organisms, and these genes have lower CAI (shaded genes in Table 2). The COA plots of *Eco, Bsu* and *Spn* show that all the Trp pathway genes cluster separately from the HEGs. From these results, it can be inferred that the Trp pathway genes are generally highly expressed throughout all the lineages of bacteria and archaea, though few genes –*trpC* in *Mlo*, *trpG* in *Hpy* and *trpF* in *Mlo* and *Pae* and all the trp genes in *Eco, Bsu* and *Spn* show differential clustering.

Relative amino acid usage (RAAU) of Trp pathway genes

Biosynthetic cost of an amino acid is considered as the total sum of the energetic cost invested in the phosphate bonds in ATP and GTP molecules and the number of available hydrogen atoms carried in NADH, NADPH, and FADH2 molecules to metabolize an amino acid (Akashi and Gojobori 2002).Tryptophan, coded by a single codon (UGG), is a rarely used amino acid having the highest molecular weight and highest biosynthetic cost (Akashi and Gojobori 2002). Figure 5 compares the usage of Tryptophan in the whole genome (WG) and that in the Trp pathway genes, in all the organisms studied. Across all phyla, compared to its usage in WG, it is used very rarely ($\ll$2 %) in the pathway genes, with the exception of *trpF* in *Mtu* (>2 %), and *Sco* and *Bsu* (both >1.5 %), and *trpG* in *Cbu* and *Hpy.*

Highly expressed proteins show increased abundance of energetically less costly amino acids, and avoid the usage of heavy amino acids (Seligmann 2003). A- or T-rich codons are known to encode for more costly amino acids (Akashi and Gojobori 2002). Hence amino acids encoded by AT-rich codons (Phe, Tyr, Met, Ile, Lys, Asn) and those encoded by GC-rich codons (Gly, Ala, Arg, Pro) do not

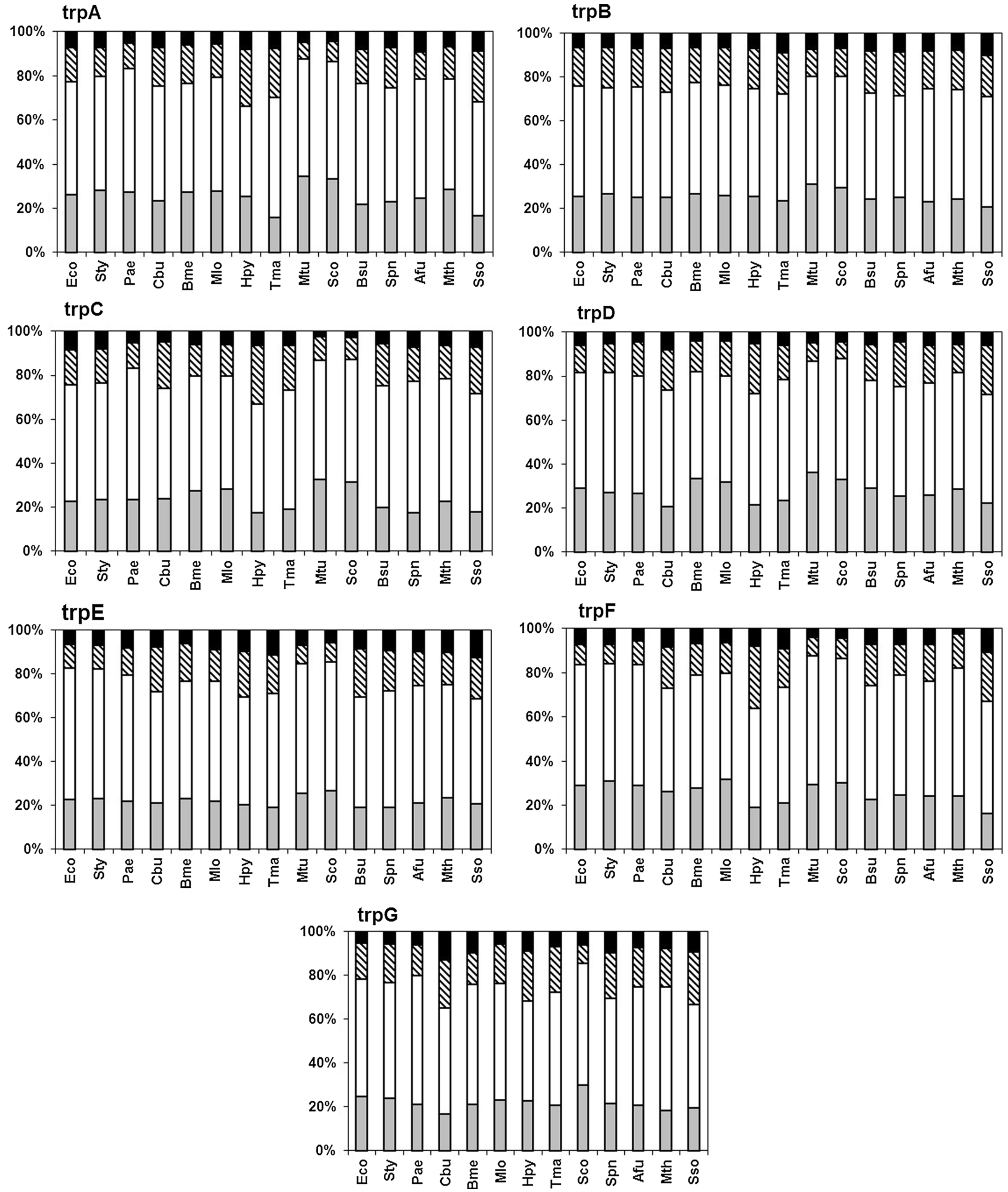


Fig. 6 Relative amino acid usages by different organisms for Trp pathway genes. The energy cost in terms of ATP molecules required for the amino acids are shown in bracket as *Black square* High (>50)—Trp, Phe, Tyr, *striped* Medium (30–40)—His, Met, Ile, Lys; *open square* Low (12–30)—Asp, Asn, Glu, Gln, Thr, Pro, Val, Cys, Arg, Leu, *gray square* Very Low (11–7)—Ala, Gly, Ser

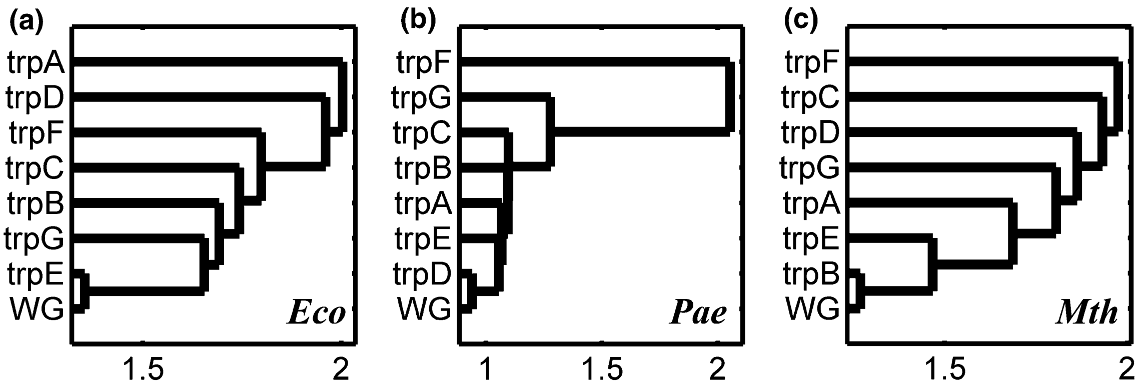


Fig. 7 Cluster Analysis on Normalized RSCU of Trp pathway genes and their WG in different organisms. See text for details

show common trends related to gene expression. In Fig. 6, the percentage use of all the amino acids are shown by grouping them according to their biosynthetic cost for each gene of the pathway in all organisms. Around 55 % of total amino acid usages in the Trp pathway genes in all organisms are contributed by the low cost amino acids (Alanine, Glycine, Serine, Glutamine, Valine, Arginine, Leucine). Alanine is most abundantly used in the maximum number of pathway genes in most of the organisms (*Eco, Sty, Pae, Bme, Mlo, Mtu, Sco*), Valine is the most abundant in all pathway genes in *Pae, Tma, Mtu, Sco, Spn, Bsu and Afu.* Abundance of medium and higher cost amino acids (Lysine, Isoleucine, and Tyrosine) increases in the organisms with lower GC content (*Cbu, Hpy, Bsu, Spn, Sso*), because these amino acids are coded by AT rich codons. Usage of heavy and energetically most costly (>50 ATP) amino acids (Phenylalananine, Tyrosine, Tryptophan) by the *trp* genes was found to be very less (<2 %) in almost all organisms. Thus it is clear from Fig. 6 that the Trp pathway genes, in general, support the principle of cost minimization of amino acid usage in organisms where the genes are highly expressed, yet show extensive variability in the usage of amino acids indicating a lack of evolution through conserved vertical descent alone.

Variation of codon usage within Trp operon genes in different organisms

As mentioned earlier, every organism has a preferred set of codons, for each amino acid, that occur most frequently in genes that are translated at high abundance. We performed cluster analysis of the normalized RSCU for the Trp pathway genes, along with their whole genome, to reveal the similarities/dissimilarities in the codon usage pattern in different organisms. Figure 7a–c show the dendrograms of all pathway genes and its whole genome (WG), based on their RSCU values, revealing the inter-genic codon usage similarities/differences in three representative organisms. In 5 out of 9 organisms (*Eco, Sty, Hpy, Mtu* and *Tma*), having the fused-genes (*trpGD* or *trpCF*) in the pathway, *trpA* forms a distinct out-group, indicating that its codon usage is different from the other pathway genes and its whole genome (WG). This is shown in Fig. 7a. The organisms (*Bsu, Pae, Spn, Mth, Sso*) where all the pathway genes are in split condition i.e., *trpE-G-D-F-C-B-A*), the genes *trpC*, *trpF*, and *trpG* always form out-groups (Fig. 7b). For its codon usage preference, *trpE* clustered with its whole genome in 5 (*Sty*, *Eco, Bme*, *Mtu, Bsu,* and *Mth*) out of 15 organisms studied (Fig. 7c). This gene-specific analysis clearly shows that variability in codon usage pattern is observed in genes within the pathway in each organism, and there is no clear-cut relationship with respect to the classification of the microorganisms as is shown in their phylogenetic tree (Fig. 1).

Variation of codon usage in different organisms for each Trp pathway

In order to find if particular genes in the Trp pathway have similar codon usage pattern in different organisms, the RSCU of 59 codons of each gene in 15 organisms were subjected to Principal Component Analysis (PCA). Figure 8 shows the principal component plot (PC1 vs PC2) of organisms for two representative Trp pathway genes—*trpB* and *trpE*. Organisms with similar loadings on PC1 and PC2 are encircled. The organisms *Eco-Sty-Bme* and *Sco-Pae-Mlo-Mtu* group together (Fig. 8b) for all pathway genes (*trpA, trpB, trpCF, trpGD,* and *trpE*). For most genes *Tma-Afu-Mth* also were found to be in close proximity, and *Hpy-Bsu-Cbu* always grouped together (Fig. 8a). The three unusual groups, different from the 16S rRNA tree (Fig. 1) (in Xie et al. 2003a, b; Woese 2000) are *Tma-Afu-Mth*, *Mlo-Pae*, and *Hpy-Cbu-Bsu*, where *Tma* from the bacterial group cluster with *Afu,* an archaea for most Trp pathway genes. It has been estimated that 24 % of the genes of the bacteria hyperthermophile *Thermotoga maritima* (*Tma*) are

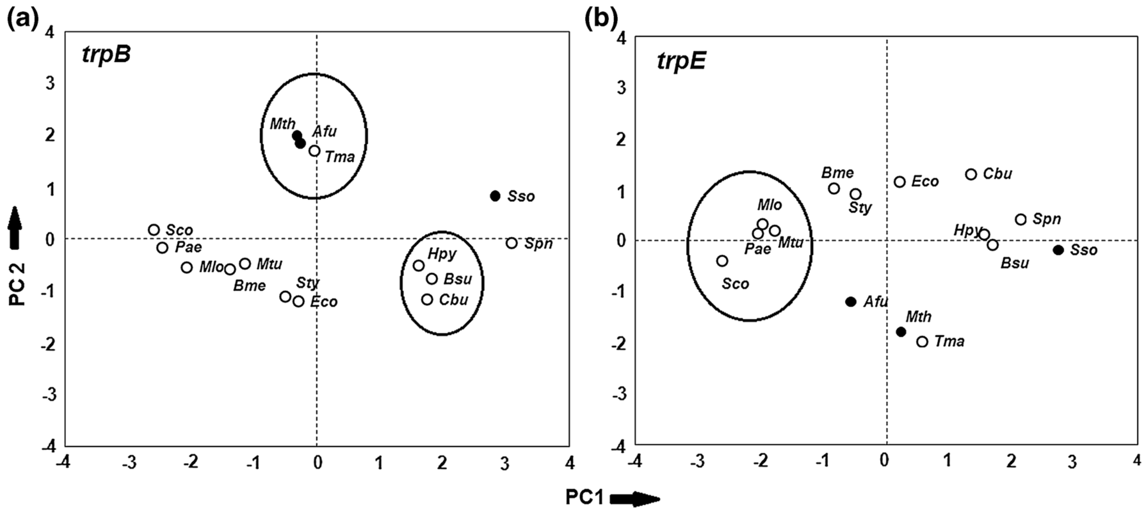


Fig. 8 Principal Component plots of codon usage of organisms for different Trp pathway genes **a** *trpB*, **b** *trpE*. *Open* and *filled circles* represent Bacteria and Archaea respectively. Organisms with similar loadings are *encircled*

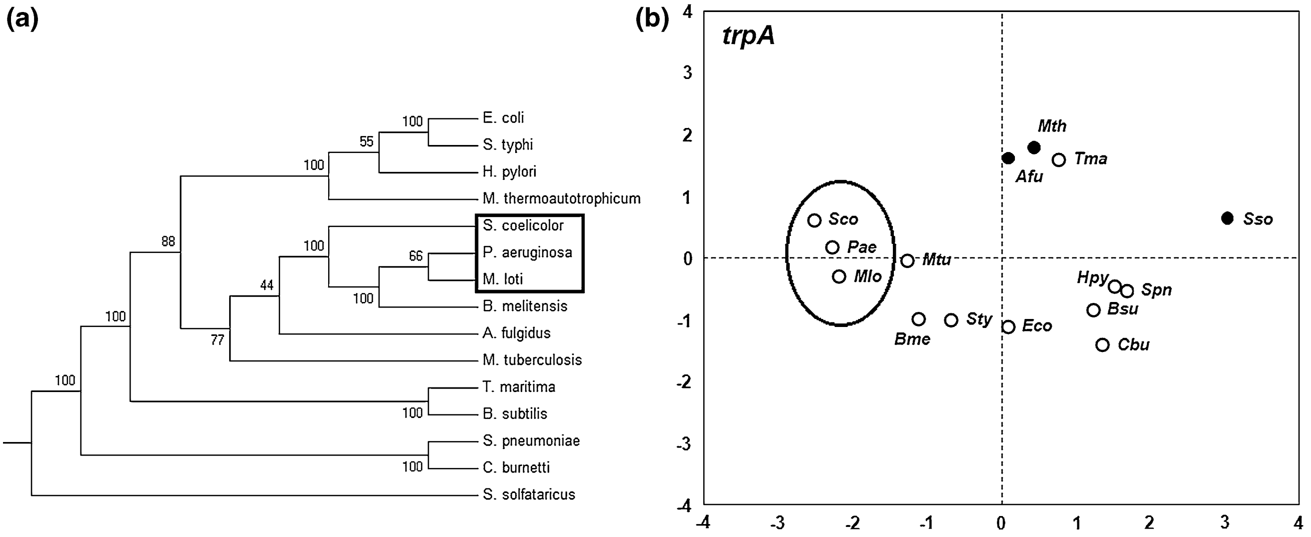


Fig. 9 **a** *trpA* gene tree, **b** PCA plot of *trpA*

similar to archaeal genes and has been derived through horizontal gene transfer (Garcia-Vallve et al. 2000; Nelson et al. 1999), and our results on Trp pathway genes seem to provide evidence for gene transfer between Archaea and Bacteria. Similarly, *Hpy, Bsu* and *Cbu* are from two different bacterial groups but in terms of their codon usage, they separate out from their usual taxonomic grouping and show similarity with each other for all the Trp genes.

Sco, Pae and *Mlo* cluster together in the phylogentic trees of *trpA, G* and *D* and in the PCA of the same, but this clustering is not seen in the 16 s rRNA tree suggesting that the PCA based on the RSCU values do show clustering similar to that of gene trees. An example is shown in the Fig. 9a in which the gene tree of *trpA* and the PCA of the RSCU values (Fig. 9b) of the *trpA* show similar grouping for some organisms, e.g., *Sco-Pae-Mlo*. This group is also seen in other genes of the pathway in both phylogenetic tree and RSCU based groups (see Fig. 9). The archaea *Sso* is dissimilar in all features—phylogenetic and codon usages. Thus, the composition based features can also show differential grouping in organisms for the same pathway genes that indicate different evolutionary trends within pathway genes.

Discussion

Microevolution in prokaryotes is commonly regulated through the acquisition and loss of genetic material by several mechanisms, the most important being the horizontal/

lateral gene transfer (H/L-GT). Even though they have been repeatedly linked with adaptation of lineages to new habitats, lifestyles, and pathogenicity, exceptions to the rules are also found in large abundance (Beiko et al. 2005; Dagan and Martin 2007; Ge et al. 2005). A bioinformatic study recently showed that a massive scale LGT may have transformed a strictly anaerobic, chemolithoautotrophic methanogen into the heterotrophic, oxygen-respiring, and bacteriorhodopsin-photosynthetic haloarchaeal common ancestor (Nelson-Sathi et al. 2012). Thus a clear-cut categorization between vertical and lateral transmission at the individual gene level is not always possible.

The relative impact of vertical transmission and lateral entry in microbial evolution is slowly giving way to the idea of extensive gene reorganization, even within groups of genes that belong to a set that perform a single function—say, production of an amino acid. Tryptophan, biochemically the most expensive amino acid to be synthesized in the cell, shows highly conserved reaction chemistry in all organisms. However, even though its biosynthetic pathway genes are frequently organized in whole-pathway operons, they have shown extensive fusion/splitting events for the same enzymes. Such an attribute is expected to facilitate multi-gene transfer in a single step. This has rendered the Trp pathway not only to be a good model system for studies in pathway evolution, it has also been used as a model system to assess the relative impact of the events of lateral gene transfer in an overall context of vertical genealogy (Xie et al. 2003a, b). The whole pathway *trp* operon analyses was also done using both phylogenetic and parametric approaches on 47 complete-genomes of *Bacteria* (Xie et al. 2004). The variety and frequency of rearrangements in the *trp* operons involving events of gene shuffling, fusion, operon splitting, total gene dispersal, and insertion of seemingly unrelated genes, raises questions about the forces of selection that enforce stability of the pathway operation. In this study, we have used a combination of comparative genomics and statistical analyses on a smaller, but carefully chosen, set of bacterial and archaeal genomes to identify signatures of diversification of genes within the Trp pathway. We selected organisms from prokaryote lineages that show *trp* gene organizations that have five pathway enzymes coded by 5–7 genes, which combine in different ways (through gene fusion and splitting) to facilitate Tryptophan biosynthesis. As has been shown in other studies (Moszer et al. 1999; Mrazek and Karlin 1999; Nakamura et al. 2004), to identify likely acquired sequences through lateral gene transfer, we have used four approaches—base composition analysis (AT, GC, and GC3 content), gene expressivity pattern, amino acid usage pattern, and codon usage patterns of the pathway genes that differ from the genomic norm of the organisms.

Our analysis shows that even though the Trp pathway genes follow the general trend of G+C content and is in equilibrium with the organism's whole genome, there is differential GC3 bias observed independent of their taxonomic grouping—higher GC3 bias in *Sco, Pae, Mlo* and lesser GC3 bias in *Sso, Cbu, Spn*. The *trp* genes being part of organism's regulatory and metabolic genes, are highly expressed in terms of their codon usage pattern in most organisms (*Pae, Cbu, Bme, Mlo, Hpy, Tma, Mtu, Sco, Afu, Mth, Sso* with CAI > 0.5). This reflects that they are highly adaptive to the codon usage pattern of the reference set of HEGs in their genomes. On the other hand, the codon usage pattern of the pathway genes in *Eco, Bsu and Spn* were found to be less adaptive (CAI < 0.5) and they exhibit moderate expression. The COA plots corroborate these results, where *trp* genes were found be clustering together with HEGs in all the organisms except *Eco, Bsu and Spn*. The principle of cost minimization in amino acid usage is supported, in general, by all pathway genes in all organisms, supporting the abundance of energetically less costly amino acids with proportional increase in the level of expression of the genes (Akashi and Gojobori 2002).

Cluster Analysis revealed that pathway genes, though work in tandem for functioning of the pathway, can evolve differentially. More variability is observed in genes, which are partners in gene fusion/splitting events (*trpC, F, G, D*). Our observations indicate that the events of gene fusion and gene splitting in the operon organization play a key role in promoting the variation in shaping the codon usage pattern in course of evolution of the pathway. The Principal Component Analysis of the codon usage patterns for all pathway genes consistently grouped *Eco-Sty-Bme* and *Sco-Pae-Mlo-Mtu* together. This is in accordance with the classification given by Xie et al. (2003a, b, 2004). But the two unusual groupings revealed from our multivariate analysis are—*Tma-Mth-Afu* and *Bsu-Cbu-Hpy*. These results clearly indicate that bacterial genomes are extremely dynamic and are continuously evolving to acquire novel ecological and pathogenic characters from distantly related species, thereby promoting diversification and speciation through lateral gene transfer (Ochman et al. 2000).

By taking microbial systems with different number of genes regulating the Trp biosynthetic pathway, we have attempted to show the variability in the pathway genes using a number of approaches. These are based on compositional features, such as base composition, usage of amino acids in the enzymes, expressivity, codon usage bias, etc. We have shown that the evolution of different genes in the same pathway in different organisms can exhibit organismal classifications that may not match with that of the 16S rRNA phylogenetic tree. Similar conclusions have been shown for large classes of organisms using phylogenetic methods (Jardine et al. 2002; Merrino et al. 2008; Nelson-Sathi et al. 2012). Our results corroborate the view that, even at the level of the genes regulating a pathway (operon module), a simple bifurcating tree of microbial phylogeny is an inadequate metaphor to represent the process of evolution (Doolittle 2004). With the bioinformatic and statistical analysis on codon usage data, we have quantified the genetic variation, which in turn can offer a different evolutionary relationship between organisms. Our overall analysis endorses a "synthesis" (Gogarten et al. 2002; Xie et al. 2003a, b; Kurland 2005) that acknowledges both the traditional vertical genealogy (tree-like behaviour) and web-like, reticulate behavior with lateral gene transfer for the evolution of Trp pathway genes. Studies that combine both phylogenetic and composition-based analyses form the basis for moving from single gene evolution to single pathway evolutionary studies, and finally to the evolution of complex network of intracellular biochemical pathways that regulate cellular functions at the systems level.

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