Research article

Comparison of theoretical proteomes: Identification of COGs with conserved and variable pI within the multimodal pI distribution
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Abstract

Background: Theoretical proteome analysis, generated by plotting theoretical isoelectric points (pI) against molecular masses of all proteins encoded by the genome show a multimodal distribution for pI. This multimodal distribution is an effect of allowed combinations of the charged amino acids, and not due to evolutionary causes. The variation in this distribution can be correlated to the organisms ecological niche. Contributions to this variation maybe mapped to individual proteins by studying the variation in pI of orthologs across microorganism genomes.

Results: The distribution of ortholog pI values showed trimodal distributions for all prokaryotic genomes analyzed, similar to whole proteome plots. Pairwise analysis of pI variation show that a few COGs are conserved within, but most vary between, the acidic and basic regions of the distribution, while molecular mass is more highly conserved. At the level of functional grouping of orthologs, five groups vary significantly from the population of orthologs, which is attributed to either conservation at the level of sequences or a bias for either positively or negatively charged residues contributing to the function. Individual COGs conserved in both the acidic and basic regions of the trimodal distribution are identified, and orthologs that best represent the variation in levels of the acidic and basic regions are listed.

Conclusion: The analysis of pI distribution by using orthologs provides a basis for resolution of theoretical proteome comparison at the level of individual proteins. Orthologs identified that significantly vary between the major acidic and basic regions maybe used as representative of the variation of the entire proteome.

Background

A protein’s Isoelectric point (pI) – the pH at which a protein has no net charge – is the basis for its isolation using isoelectric fociussing and along with Molecular Mass (Mr) is exploited in two dimensional gel electrophoresis used to separate a cell’s protein content. Bjellquist [1,2] has shown that a the pI of a denatured linear protein can be calculated with high accuracy using the pK values of the amino acids responsible for charge. Using these calculations, it is possible to create an image of an organisms
Exhaustive work on virtual proteomes has been completed recently by two groups. Weiller et al [5] have proved that the multimodal distribution of protein pl is present in randomly generated sequences, and is a function of allowed combinations of amino-acid pKa values, rather than a cause of sequence evolution. A trimodal distribution is present in the virtual proteome of most organisms, with minima at 7.4 and 8.0. Knight et al [6] have shown that the variation in proteomes though the trimodal distribution is largely maintained – is influenced by the ecological niche of the organism.

Environmental influences of the proteome are known[7]. Amino acid usage is influenced by the G+C content of a genome [8]. Acidic residues predominate over basic residues in halophilic bacteria [9,10], and compositional properties are further distinguished in thermophilic and mesophilic bacteria – with a preference for salt-bridges (residues with opposite charges) and long-chain hydrophobic residues in the former for increased stability [11].

All studies so far have used the gross properties of the proteome, or broad functional groups (e.g membrane proteins), and not attempted to resolve the multimodal distribution on the basis of individual proteins. This analysis could provide answers to the apparent conflict that though the pl multimodal distribution is caused by the properties of amino acids and not evolutionary factors, environmental influences induce variation in the sizes of each cluster in the distribution. By mapping the variation of pl in orthologs, one can in principal identify proteins whose pl is conserved as well as those whose pl does not seem to be responsible for its function, and whose variation maybe used as markers for an organism’s environment.

The COG (Cluster of Orthologous Groups) database [12,13] lists orthologs present across completed genomes. In this study, we consider the subset of an organism’s proteome, as specified by the COG database, which contain orthologs present in other genomes – providing a basis to study the variation of pl of individual proteins across genomes.

Results and discussion

Comparison of virtual proteomes among different Bacteria organisms

The predicted proteomes, using values of Mr and pl calculated from the protein ortholog sequence, all displayed a trimodal distribution, with minima at 7.4 and 8.1. For convenience, the three major peaks demarked by these minima are referred to as the acidic cluster (pl less than 7.4), “neutral” cluster (pl between 7.4 and 8.1) and basic cluster (pl greater than 8.1). This observation of a trimodal distribution is consistent with earlier results calculated from the complete genome [4-6], showing that orthologs maybe used as representative samples of the complete genome. Figure 1 shows representative plots of proteomes using whole genomes and ortholog sub-sets for Escherichia coli K12 and Helicobacter pylori, Buchenara and Halobacterium. As only orthologs are used, it is possible to compare the pl of two genomes, by using a scatter plot. Diagonal points show invariant pl between the organisms, while a shift in pl is visible as an off-diagonal point. As the trimodal distribution is dominated by the large acidic and basic clusters, A shift in the pl from one cluster to the other is visible on the upper left and lower right quadrants of the plot. Figure 2 shows the pairwise comparison of the pl and Mr for the proteomes of two variants of E. coli (K-12 and 0157), between E. coli K12 and H. pylori which have different environmental pl – and extremophiles Buchenara and Halobacterium. Closely related variants of the same species do not have much variation in both pl and Mr, however the effect of a changed pH environment – specifically the acidic stomach for H. pylori and the basic intestine for E. coli – causes a large proportion of orthologs to change pl to compensate for the external pH change. Organisms that have extreme proteome pl distributions are Halobacterium – which has a large acidic cluster and Buchnera – a large negative cluster. As expected, most orthologs shared by the two organisms show a shift in their pl from one cluster to the other, however a baseline of pl conservation is maintained, implying that the pl maybe a conserved property for a few orthologs. The Mr is much more highly conserved, with the scatter plot clustered along the diagonal.

Variation in pl among functional categories of COGS

The COG database is functional classified into eighteen categories [12] (Table 1, shows a listing of these functions, which the single letter code used in the figures). The distribution of pl values across all organisms for each function, summarised by the mean and standard deviation, is shown in Figure 3. The mean values for each function is close to neutral pH, with a deviation spreading across
Figure 1
Ortholog (left) and whole genome (right) isoelectric point (pI) frequency distributions for (A) Escherichia coli K-12, (B) Helicobacter pylori, (C) Buchenera APS and (D) Halobacterium sp. NRC-1.
Figure 2
Pairwise comparison of isoelectric point and molecular mass of orthologs. (A) Escherichia coli K12 and 0517; (B) Escherichia coli K12 and Helicobacter pylori (C) Halobacterium sps NCR-1 and Buchnera sps APS
both clusters. Again the use of orthologs provides the means to correlate conservation of pl between pairs of organisms. The mean pairwise-correlation of ortholog pl corresponding to each function was also computed (Figure 4A), along with the corresponding pairwise correlation of ortholog Mr (Figure 4B). We have used Kruskal-Wallis multiple comparison testing [14] to identify groups that significantly deviate from the expected distribution. Five functional groups deviate significantly, three towards the basic cluster and two towards the acidic cluster (Table 1).

Membrane proteins are known to have a preference for the basic cluster, caused by the larger proportion of basic charged residues to compensate for the negatively charged membrane bilayer [4,6]. A functional requirement for either basic or acidic charges could thus influence the protein's pl. Orthologs, by definition, perform the same function in different organisms, and if preference for either basic or acidic charged residues is related to this function, this should be reflected in the protei\n's pl having a bias for the respective cluster. However, the conservation of pl maybe accidental, especially if the proteins are highly conserved. We have calculated the pairwise distance of all proteins within a COG as a measure of their sequence similarity. The distributions of distance for each functional group is shown in Figure 4C.

Proteins associated with the group "J", involved with translation, ribosomal structure and biogenesis, are dominated by highly conserved ribosomal proteins – and this high level of conservation and intolerance to mutation is reflected in an invariance of pl. This conservation is also reflected in the higher correlation for this group of proteins in their Mr. Other groups which are polarised to either the acidic or basic modes of the pl distribution do not show such a high level of conservation, and pl conservation is dictated by the nature of their function.

**Table 1: Variation in pl in functionally classified groups of COGs.** The table lists functional groups of COGs as defined in the COG website [22] along with the results of the Dunn Multiple Comparison test. $R_i$ – mean rank for the group, $R_t$ – mean rank for sample representing the complete distribution. Significant values are those where $|R_t - R_i|$ > 467.35 which is calculated for $\alpha = 0.2$

| Metabolism                                                                 | $R_i$ | $R_t - R_i$ |
|---------------------------------------------------------------------------|-------|-------------|
| C Energy production and conversion                                         | 2563.72 | 276.96      |
| E Amino acid transport and metabolism                                      | 2792.99 | 47.69      |
| F Nucleotide transport and metabolism                                      | 2191.12 | 649.56      |
| G Carbohydrate transport and metabolism                                    | 2958.98 | -118.3     |
| H Coenzyme metabolism                                                     | 2736.4 | 104.28     |
| I Lipid metabolism                                                        | 2587.87 | 252.81     |
| Q Secondary metabolism biosynthesis, transport and catabolism             | 2479.07 | 361.62     |

| Information storage and processing                                         | $R_i$ | $R_t - R_i$ |
|---------------------------------------------------------------------------|-------|-------------|
| J Translation, ribosomal structure and biogenesis                          | 3399.31 | -558.63    |
| K Transcription                                                           | 2977.93 | -137.25    |
| L DNA replication, recombination and repair                                | 3530.56 | -689.88    |

| Cellular process                                                           | $R_i$ | $R_t - R_i$ |
|---------------------------------------------------------------------------|-------|-------------|
| M Cell Envelope biogenesis, Outer membrane                                 | 3287.13 | -446.45    |
| N Cell motility and secretion                                              | 2722.08 | 118.6      |
| O Post translation modification, protein turnover, chaperones              | 2445.01 | 395.67     |
| P Inorganic ion transport and metabolism                                   | 3482.93 | -642.25    |
| T Signal transduction mechanisms                                           | 2219.33 | 621.45     |
| D Cell division and chromosome partitioning                                | 2836.43 | 4.25       |

| Poorly characterized                                                      | $R_i$ | $R_t - R_i$ |
|---------------------------------------------------------------------------|-------|-------------|
| R General function prediction only                                         | 3189.18 | -348.5     |
| S Function unknown                                                        | 2918.88 | -78.2      |

$R_t = 2840.68$
COGs 1689, 3016, 3783 and 3801, however their frequency of occurrence among all organisms is less than ten percent. This is an expected result as the pI distribution is caused by an interplay of acidic and basic residues which account for the large acidic and basic clusters. The "neutral" cluster is not caused by the absence of charged residues but because allowed pK combinations of charged residues are minimum at pH 7.1 and 8.4 [5].

Proteins conserved in the acidic and basic clusters however have a preponderance of acidic and basic residues respectively, which maybe required for their function. A complete list of COGs whose protein pI are conserved in these clusters is listed as an additional file. We have scaled the frequency of conservation by the frequency of occurrence, so that only COGs which are maximally represented across the organisms in the dataset are used as markers of each cluster. These are listed in Table 2 and Table 3 respectively. The dominant groups of proteins which seem to require an acidic pI are the amino acid tRNA synthetases. Among those proteins whose pI is highly conserved in the basic cluster are a large number of ribosomal proteins. Although highly conserved and intolerant to mutation, ribosomal proteins interweave with negatively charged RNA to form the ribosome, and being positively charged will be a requirement for strong electrostatic interactions.

A majority of COGs however show distributions across both the basic and acidic clusters. Knight et al, have shown a correlation to a change in proteome patterns with the organism's ecological niche. Since the proteome always exists in a trimodal distribution, it will only vary from organism to organism in the relative amounts of proteins which are present in each of the three clusters of the pI distribution. We have computed the probability of being in both the acidic and basic clusters weighted by the frequency of occurrence in the organisms under consideration in order to identify COGs which are highly represented and show no particular preference for either cluster. This list of COGs, with a joint probability greater than a cutoff value of 0.6 is tabulated in table 4. For reference, the individual values of genes corresponding to the four organisms is also listed, and in a majority of cases show good agreement with the shift in an organism's total

Figure 3
Frequency distribution for COG mean pI (grey vertical bars). The variation of pI (mean and standard deviation) for functionally classified groups of COGs is overlayed. Functional groups denoted by single letters is expanded in Table 1
theoretical proteome towards either the basic or acidic clusters. Except for some ribosomal proteins, which appear on the list because of their high frequency of occurrence, all other COGs are membrane-based proteins, which would have direct contact with the external environment. These COGs best represent an organism's shift from expected levels of the acidic and basic clusters of the multimodal distribution, and it is possible that they may be used as markers to predict an organism's ecological niche, with particular reference to its environmental pH in free living microorganisms.

Extrapolating results obtained from using the theoretical proteome must be viewed with caution as predicted pI values are for unfolded proteins obtained from sequence and not the native folded proteins in the cellular microenvironment, which remain unknown. We have resisted attempting to correlate observations of an ortholog's theoretical pI with its function, unless clearly obvious, for this reason. The theoretical proteome is also generated from the total set of proteins present in an organism's genome, while only a subset may be expressed at any given time and will vary in response to external stimuli. An organism may also respond to evolutionary pressure such as environmental pH by increasing the number of copies of a charged protein, a ploy frequently adopted in drug resistance. The effect of a shift in the relative levels of the acidic and basic peaks may be replicated by an increase in copy number of proteins belonging to the relevant cluster. As theoretical proteome studies are not weighted by the copy number of the individual proteins, for lack of relevant data related to the quantity of individual proteins for the entire proteome, these observations are impossible to make.

**Conclusion**

Analysis of ortholog pI across forty-two microorganism genomes which contain representatives of free living archaea and bacteria are analysed to identify orthologs which are invariant in pI as well as those amenable to changes in pI. Orthologs with a high frequency of occurrence and variation in pI are shortlisted, and may be used as markers in future studies which attempt to map proteome properties to variation in the organisms ecological niche.

**Methods**

Genome sequences and the COG database generated with 43 organisms with sequences and alignments were sourced from NCBI [15]. *Saccharomyces cerevisiae* was removed from the dataset so that it contained only archaea and bacteria which were single-celled organisms. This was intentionally to include only genomes of organisms which would have their membrane directly in contact with the external environment and included a range of organisms with diverse ecological niches.
The isoelectric point is determined from an equation subtracting the sum of all negative charges (of all acidic residues) from the sum of all positive charges (of all basic residues), by varying the pH by bisectional nesting of intervals on the pH scale until the interval is smaller than a given level (0.001), as described in [2]. Fixed values for pK are considered to calculate the isoelectric point, and are the same as used in the molecular weight/isoelectric point program at [http://www.expasy.org](http://www.expasy.org)[16].

The molecular weights and isoelectric point of all the predicted open reading frames from the completed genomes of a number of prokaryotes were generated. All ORFs that are classified by respective COG identities and assigned to one of the 18 functional groups [12] were used in comparative analysis between genomes.

Correlation of molecular weights and pI were performed, using scripts written in house, for all COGs classified to a particular function for each pair of organisms. Pairwise distances were calculated for all pairs of proteins belonging to the specified COG. Sequences assigned to each COG were aligned with ClustalW [17] using default parameters, and the resulting multiple alignment used to generate a distance matrix with the program PROTDIST, using default parameters (Jones-Taylor-Thornton model, where the distance is scaled in units of the expected fraction of amino acids changed), from the Phylip package [18]. Perl scripts used to automate this protocol were written using BioPerl Modules [19].

Individual molecular weight and isoelectric point along with pairwise distances were stored in a MySQL database v 4.1.9, running on a Fedora Core Linux dual Xeon Server, to enable easy retrieval. Statistical properties on the data

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**Figure 5**

Isoelectric point distribution of orthologs sorted by mean isoelectric point value. Black dot – mean value for COG, grey bar – standard deviation for COG, red point – pI value of individual genes classified under the COG. The sorted list of COG’s used for the X-axis is available as an additional file.
(mean, variance and correlation coefficients) were calculated using standard methods after sectioning the data at the levels of COGs and functional groups. Figures plotted in this paper were made using Sigmaplot (Systat Software Inc, Richmond, California, USA).

Statistical significance of functional groups was assessed using non-parametric Kruskal-Wallis tests and Dunn's Multiple Comparison [20] tests (alpha = 0.2), after randomly sampling three hundred sequences from each group, and from the total population to form the sample sets.

**Table 2: COGs conserved in the acidic cluster of the multimodal distribution.** P(a) and P(b) are the frequency of occurrence in the acidic and basic clusters respectively, F is the function group. The COGs are listed with their P(v) which is score of their variability between the acidic and basic clusters of the pI distribution weighted by their frequency of occurrence across all genomes in the dataset. pI values of four organisms – the extremophiles Buchnera (environment bacteriocyte), Halobacterium (highly acidic – high salt), and E. coli and H. pylori – intestinal bacteria with different environmental pH.

| COG     | P(a) | P(b) | P(v) | F                  | Hbs | Eco | Hpy | Buc |
|---------|------|------|------|-------------------|-----|-----|-----|-----|
| COG2890 | 0.80 | 0.18 | 0.35 | J Predicted rRNA or tRNA methylase | 4.23 | 4.99 | 5.61 | 9.38 |
| COG0201 | 0.81 | 0.00 | 0.05 | G Transketolase     | -   | 5.61 | 6.23 | 7.98 |
| COG0215 | 0.82 | 0.11 | 0.24 | J Cytochrome-tRNA synthetase | 4.47 | 5.29 | 5.88 | 9.33 |
| COG0016 | 0.82 | 0.14 | 0.32 | J Phenylalanine-tRNA synthetase alpha subunit | 4.14 | 5.71 | 6.35 | 9.51 |
| COG0449 | 0.82 | 0.04 | 0.08 | M Glucosamine 6-phosphate synthetase, contains amidotransferase and phosphosugar isomerase | 5.08 | 5.52 | 6.05 | 9.52 |
| COG0206 | 0.82 | 0.08 | 0.16 | D Cell division GTPase | 4.35 | 4.63 | 5.26 | 5.08 |
| COG0436 | 0.82 | 0.04 | 0.10 | E PLP-dependent aminotransferases | 4.41 | 6.15 | 7.41 | -   |
| COG0468 | 0.83 | 0.15 | 0.31 | L RecA/RadA recombine | 4.44 | 5.06 | 5.47 | -   |
| COG0125 | 0.83 | 0.17 | 0.35 | F Thymidylate kinase | 4.32 | 5.26 | 8.97 | 10.01 |
| COG0084 | 0.84 | 0.03 | 0.09 | L Mg-dependent DNase | -   | 5.44 | 5.43 | 8.81 |
| COG0504 | 0.84 | 0.07 | 0.18 | F CTP synthase (UTP-ammonia lyase) | 4.47 | 5.58 | 7.55 | 9.01 |
| COG0124 | 0.86 | 0.12 | 0.27 | J Histidyl-tRNA synthetase | 4.29 | 5.57 | 5.54 | 9.28 |
| COG0443 | 0.86 | 0.02 | 0.04 | O Molecular chaperone | 3.95 | 5.08 | 5.02 | 7.44 |
| COG0024 | 0.86 | 0.08 | 0.22 | J Methionine aminopeptidase | 4.05 | 5.55 | 5.89 | 8.70 |
| COG0209 | 0.87 | 0.05 | 0.10 | F Ribonucleotide reductase alpha subunit | 4.39 | 5.89 | 5.73 | 9.11 |
| COG0142 | 0.87 | 0.06 | 0.13 | H Geranylgeranyl pyrophosphate synthase | 4.31 | 4.96 | 5.94 | 9.58 |
| COG0468 | 0.87 | 0.10 | 0.23 | L RecA/RadA recombinase | 4.35 | 5.76 | 5.93 | 8.86 |
| COG0112 | 0.89 | 0.10 | 0.19 | J Glycine hydroxymethyltransferase | 4.30 | 5.95 | 6.41 | 9.22 |
| COG0525 | 0.88 | 0.12 | 0.23 | J Valyl-tRNA synthetase | 4.09 | 5.19 | 6.12 | 9.38 |
| COG0060 | 0.89 | 0.11 | 0.23 | J Isocitrate-tRNA synthetase | 4.13 | 5.63 | 6.15 | 9.32 |
| COG0073 | 0.89 | 0.09 | 0.17 | R EMAP domain | 4.04 | 5.29 | 5.42 | 8.90 |
| COG0172 | 0.89 | 0.09 | 0.21 | J Seryl-tRNA synthetase | 4.60 | 5.30 | 6.70 | 9.42 |
| COG0018 | 0.89 | 0.09 | 0.20 | J Arginyl-tRNA synthetase | 4.18 | 5.29 | 5.94 | 9.72 |
| COG0143 | 0.89 | 0.09 | 0.20 | J Methionyl-tRNA synthetase | 4.16 | 5.39 | 6.00 | 9.27 |
| COG1109 | 0.89 | 0.09 | 0.21 | G Phosphomannomutase | 4.43 | 5.55 | 6.18 | 9.03 |
| COG0006 | 0.90 | 0.04 | 0.11 | E Xaa-Pro aminopeptidase | 4.42 | 5.35 | 5.73 | -   |
| COG0442 | 0.91 | 0.09 | 0.18 | J Prolyl-tRNA synthetase | 4.45 | 5.11 | 5.90 | 9.51 |
| COG0126 | 0.91 | 0.07 | 0.14 | G 3-phosphoglycerate kinase | 4.29 | 5.06 | 6.18 | 9.37 |
| COG0012 | 0.91 | 0.09 | 0.17 | R Predicted GTPase | 4.15 | 4.86 | 5.50 | 9.07 |
| COG0149 | 0.91 | 0.02 | 0.09 | G Triosephosphate isomerase | 4.15 | 5.55 | 7.58 | 9.23 |
| COG0492 | 0.92 | 0.08 | 0.16 | O Thioredoxin reductase | 4.27 | 5.24 | 6.02 | 9.36 |
| COG0072 | 0.93 | 0.07 | 0.14 | J Phenylalanine-tRNA synthetase beta subunit | 4.33 | 5.26 | 6.63 | 8.30 |
| COG0085 | 0.94 | 0.02 | 0.08 | K DNA-directed RNA polymerase beta subunit/140 kD subunit (split gene in Mjan, Mthe, Aful) | 4.77 | 5.14 | 6.14 | 7.81 |
| COG0231 | 0.94 | 0.02 | 0.08 | J Translation elongation factor P/translation initiation factor elf-5A | 4.78 | 5.28 | 5.34 | 9.40 |
| COG0459 | 0.95 | 0.01 | 0.04 | O Chaperonin GroEL (HSP60 family) | 4.16 | 4.84 | 5.51 | 5.04 |
| COG0013 | 0.95 | 0.05 | 0.09 | J Alanyl-tRNA synthetase | 4.29 | 5.51 | 5.96 | 9.42 |
| COG0592 | 0.96 | 0.04 | 0.09 | L DNA polymerase sliding clamp subunit (PCNA homolog) | 3.97 | 5.20 | 5.45 | 8.93 |
| COG0202 | 0.98 | 0.02 | 0.05 | K DNA-directed RNA polymerase alpha subunit/40 kD subunit | 4.11 | 4.95 | 4.95 | 5.03 |
| COG0148 | 0.98 | 0.00 | 0.00 | G Elongase | 4.38 | 5.28 | 5.42 | 6.93 |
| COG0480 | 0.99 | 0.01 | 0.03 | J Translation elongation and release factors (GTPases) | 4.41 | 5.41 | 5.23 | 7.61 |
To score the COGs both for variation and conservation, probabilities were estimated from frequencies in the dataset. The frequency of occurrence of a COG $i$, $F(i)$, calculated as

$$F(i) = \frac{n_o}{n_t}.$$  

Where $n_o$ is the number of organisms in which the COG is present and $n_t$ is the number of organisms in the sample dataset (42).

\[ P_j(i) = \left( \frac{n_r}{n_t} \right) \cdot (F(i)) \]

where $i$ is the cog, $j$ is the cluster – either acidic, neutral or basic, and $r$ the range corresponding to the cluster – 0–7.4 for acidic, 7.4–8.1 for basic, and 7.4–14 for basic. $n_r$ is the number of proteins in the given cluster, $n_t$ total number of proteins corresponding to $i$. $P_j(i)$ is the conservation score for the cluster $j$, and is the joint probability of conservation and occurrence of the COG $i$.

### Table 3: COGs conserved in the Basic cluster of the multimodal distribution. Table headers are the same as in Table 2.

| COG     | P(a) | P(b) | P(v) | F      | Role                              | Hbs | Eco | Hpy | Buc |
|---------|------|------|------|--------|-----------------------------------|-----|-----|-----|-----|
| COG0101 | 0.11 | 0.82 | 0.27 | J      | Pseudouridylate synthase (tRNA psi55) | 5.52 | 8.68 | 9.74 | 9.66 |
| COG0582 | 0.05 | 0.83 | 0.12 | L      | Integrase                          | 5.31 | 9.70 | 9.61 | -   |
| COG0080 | 0.15 | 0.85 | 0.30 | J      | Ribosomal protein L11              | 3.72 | 9.64 | 9.55 | 10.01 |
| COG0477 | 0.09 | 0.87 | 0.20 | E      | Permeases of the major facilitator superfamily | 6.54 | 9.32 | 9.14 | 9.65 |
| COG0477 | 0.09 | 0.87 | 0.20 | E      | Permeases of the major facilitator superfamily | 6.54 | 9.32 | 9.14 | 9.65 |
| COG0477 | 0.09 | 0.87 | 0.20 | E      | Permeases of the major facilitator superfamily | 6.54 | 9.32 | 9.14 | 9.65 |
| COG0477 | 0.09 | 0.87 | 0.20 | E      | Permeases of the major facilitator superfamily | 6.54 | 9.32 | 9.14 | 9.65 |
| COG0541 | 0.07 | 0.88 | 0.19 | N      | Signal recognition particle GTPase | 4.27 | 9.52 | 9.40 | 9.82 |
| COG0255 | 0.09 | 0.89 | 0.21 | J      | Ribosomal protein L29              | 4.69 | 9.98 | 9.70 | 10.45 |
| COG0092 | 0.07 | 0.93 | 0.14 | J      | Ribosomal protein S3               | 3.87 | 10.27 | 10.25 | 10.40 |
| COG0198 | 0.07 | 0.93 | 0.14 | J      | Ribosomal protein L24              | 4.37 | 10.21 | 9.86 | 10.57 |
| COG0051 | 0.04 | 0.93 | 0.11 | J      | Ribosomal protein S10              | 5.15 | 9.68 | 9.30 | 9.85 |
| COG0089 | 0.02 | 0.95 | 0.07 | J      | Ribosomal protein L22              | 4.13 | 9.94 | 10.00 | 10.00 |
| COG0146 | 0.05 | 0.95 | 0.09 | J      | Ribosomal protein L18              | 5.13 | 10.42 | 10.16 | 10.94 |
| COG0087 | 0.02 | 0.98 | 0.05 | J      | Ribosomal protein L3               | 5.57 | 9.90 | 10.24 | 10.60 |
| COG0191 | 0.02 | 0.98 | 0.05 | J      | Ribosomal protein L22              | 4.83 | 9.72 | 9.43 | 10.19 |
| COG0098 | 0.02 | 0.98 | 0.05 | J      | Ribosomal protein L5               | 5.08 | 10.23 | 11.26 | 10.85 |
| COG0120 | 0.08 | 0.98 | 0.05 | J      | Ribosomal protein L15              | 4.92 | 10.11 | 9.90 | 10.51 |
| COG0121 | 0.08 | 0.98 | 0.05 | N      | Preprotein translocase subunit SecY | 5.56 | 9.89 | 9.87 | 9.72 |
| COG0049 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein S7               | 5.51 | 10.37 | 10.17 | 10.33 |
| COG0081 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein L1               | 4.13 | 9.64 | 9.61 | 9.86 |
| COG0094 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein L5               | 4.66 | 9.49 | 9.74 | 9.89 |
| COG0096 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein S8               | 4.85 | 9.44 | 9.79 | 9.74 |
| COG0097 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein L6               | 4.20 | 9.71 | 9.77 | 10.07 |
| COG0099 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein S13              | 4.52 | 10.78 | 10.23 | 10.54 |
| COG0100 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein S11              | 5.72 | 11.33 | 10.33 | 11.18 |
| COG0102 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein L13              | 4.50 | 9.91 | 9.82 | 9.93 |
| COG0104 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein S15P/S13E        | 4.83 | 10.40 | 10.00 | 10.17 |
| COG0186 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein S17              | 4.75 | 9.64 | 10.01 | 10.14 |
| COG0252 | 0.05 | 0.98 | 0.04 | J      | Ribosomal protein S4 and related proteins | 4.81 | 10.05 | 10.08 | 10.00 |
| COG0199 | 0.02 | 0.98 | 0.04 | J      | Ribosomal protein S14              | 5.86 | 11.16 | 11.01 | 11.38 |
| COG0048 | 0.00 | 1.00 | 0.00 | J      | Ribosomal protein S12              | 9.91 | 10.88 | 10.70 | 11.09 |
| COG0090 | 0.00 | 1.00 | 0.00 | J      | Ribosomal protein L2               | 9.51 | 10.93 | 10.36 | 10.77 |
| COG0093 | 0.00 | 1.00 | 0.00 | J      | Ribosomal protein L14              | 9.60 | 10.43 | 10.45 | 10.43 |
| COG0103 | 0.00 | 1.00 | 0.00 | J      | Ribosomal protein S9               | 9.39 | 10.94 | 10.70 | 11.40 |
| COG0197 | 0.00 | 1.00 | 0.00 | J      | Ribosomal protein L16/L10E         | 9.28 | 11.23 | 10.44 | 10.88 |
Table 4: Highly occurring COGs with pI varying across both the acidic and basic clusters. Table headers are the same as in Table 2.

| COG    | P(a) | P(b) | P(v) | F | Role                                                                 | Hbs | Eco | Hpy | Buc |
|--------|------|------|------|---|-----------------------------------------------------------------------|-----|-----|-----|-----|
| COG0760 | 0.29 | 0.31 | 0.60 | O | Parvulin-like peptidyl-prolyl isomerase                               | -   | 6.83| 9.04| 9.65|
| COG0725 | 0.31 | 0.29 | 0.60 | P | ABC-type molybdate transport system, periplasmic component             | 4.90| 7.53| 9.70| -   |
| COG0042 | 0.44 | 0.25 | 0.60 | R | Predicted TIM-barrel enzymes, possibly dehydrogenases, nifR3 family   | 4.82| 6.04| 9.20| -   |
| COG0275 | 0.28 | 0.46 | 0.60 | M | Predicted S-adenosylmethionine-dependent methyltransferase             | -   | 5.92| 8.99| 9.93|
| COG0258 | 0.69 | 0.30 | 0.61 | L | 5'-3' exonuclease (including N-terminal domain of PolI)                | 4.06| 5.74| 9.06| 9.59|
| COG0640 | 0.38 | 0.28 | 0.61 | K | Predicted transcriptional regulators                                  | 4.49| 8.48| -   | -   |
| COG0444 | 0.37 | 0.27 | 0.61 | E | ABC-type dipeptide/oligopeptide/nickel transport system, ATPase componen| 4.36| 6.64| 8.29| -   |
| COG0009 | 0.61 | 0.30 | 0.61 | J | Predicted transcriptional regulators                                  | 4.43| 5.36| -   | 9.52|
| COG0226 | 0.39 | 0.30 | 0.61 | P | ABC-type phosphate transport system, periplasmic component             | 4.04| 8.37| -   | -   |
| COG1475 | 0.41 | 0.26 | 0.62 | K | Predicted transcriptional regulators                                  | 4.46| 6.78| 8.39| -   |
| COG0190 | 0.51 | 0.30 | 0.62 | H | 5,10-methylene-tetrahydrofolate dehydrogenase/Methenyl tetrahydrofolate| 4.36| 5.61| 8.74| 9.70|
| COG1057 | 0.31 | 0.33 | 0.62 | H | Nicotinic acid mononucleotide adenyltransferase                        | -   | 5.38| 8.80| 9.68|
| COG0482 | 0.42 | 0.28 | 0.62 | J | Predicted tRNA methyltransferase, contains the PP-loop ATPase domain   | -   | 4.89| 8.82| 9.50|
| COG1121 | 0.29 | 0.35 | 0.62 | L | Predicted S-adenosylmethionine-dependent methyltransferase             | 4.61| 9.44| -   | 9.21|
| COG0359 | 0.31 | 0.48 | 0.62 | J | Predicted transcriptional regulators                                  | 7.18| 7.10| 9.83| -   |
| COG0226 | 0.39 | 0.30 | 0.61 | P | ABC-type phosphate transport system, periplasmic component             | 4.04| 8.37| -   | -   |
| COG1475 | 0.41 | 0.26 | 0.62 | K | Predicted transcriptional regulators                                  | 4.46| 6.78| 8.39| -   |
| COG0190 | 0.51 | 0.30 | 0.62 | H | 5,10-methylene-tetrahydrofolate dehydrogenase/Methenyl tetrahydrofolate| 4.36| 5.61| 8.74| 9.70|

Mean: 4.76 6.64 8.75 9.47
Standard Deviation: 0.7 1.14 0.73 0.69
We estimate the joint probability of variation between the acidic and basic clusters for a COG i, $\mathbb{P}(v)$, as the probability of variation into its frequency of occurrence calculated as

$$\mathbb{P}(v) = \frac{1 - \left( \frac{n^a_i - n^b_i}{n_i} \right)}{F(i)}$$

where $n^a_i$ and $n^b_i$ are the number of proteins belonging to the COG i in the acidic and basic cluster of the distribution respectively, $n_i$ being the total number of proteins classified under COG i.

Membrane proteins were predicted using TOPPRED [21].

**Additional material**

**Additional file 1**

List of COGs sorted by mean $p_l$. File with rows containing COG, mean, standard deviation and $p_l$ of individual ortholog proteins that were used in the calculation described in this paper. The rows are sorted by mean $p_l$ (column 2) and used to plot figure 5 of the manuscript. The file is tab-delimited and will open in any spreadsheet program.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-6-116-S1.CSV](http://www.biomedcentral.com/content/supplementary/1471-2164-6-116-S1.CSV)

**Additional file 2**

COGs with $p_l$ conserved in acid and basic clusters. Text file containing the comprehensive list of COGs which are conserved in either the acidic or basic cluster of the multimodal distribution.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-6-116-S2.txt](http://www.biomedcentral.com/content/supplementary/1471-2164-6-116-S2.txt)

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