Noname manuscript No.
(will be inserted by the editor)

Bacterial protein interaction networks: connectivity is ruled by gene conservation, essentiality and function.

Maddalena Dilucca · Giulio Cimini ·
Andrea Giansanti

Received: date / Accepted: date

Abstract Protein-protein interaction (PPI) networks are the backbone of all processes in living cells. In this work we relate conservation, essentiality and functional repertoire of a gene to the connectivity k of the corresponding protein in the PPI networks. Focusing on a set of 42 bacterial species with reasonably separated evolutionary trajectories, we investigate three issues: i) whether the distribution of connectivity values changes between PPI subnetworks of essential and nonessential genes; ii) how gene conservation, measured both by the evolutionary retention index (ERI) and by evolutionary pressures (evaluated through the ratio K_a/K_s and ENC plots) is related to the connectivity of the corresponding protein; iii) how PPI connectivities are modulated by evolutionary and functional relationships, as represented by the Clusters of Orthologous Proteins (COGs). We show that conservation, essentiality and functional specialization of genes control in a quite universal way the topology of the emerging bacterial PPI networks. Noteworthy, a structural transition in the network is observed such that, for connectivities $k \geq 40$, bacterial PPI networks are mostly populated by genes that are conserved, essential and which, in most cases, belong to the COG cluster J, related to ribosomal functions and to the processing of genetic information.

Keywords Protein-protein interaction · ERI · COG

M.Dilucca
Dipartimento di Fisica, Sapienza University of Rome, 00185, Rome, Italy
E-mail: maddalena.dilucca@gmail.com

G.Cimini
Dipartimento di Fisica, Tor Vergata University of Rome, 00133, Rome, Italy
Istituto dei Sistemi Complessi CNR UoS, Rome, Italy

A.Giansanti
Dipartimento di Fisica, Sapienza University of Rome, 00185, Rome, Italy
INFN Roma1 unit, Rome, Italy

1 Introduction

To operate biological activities in living cells, proteins work in association with other proteins, often assembled in large complexes. Hence, knowing the interactions of a protein is important to understand its cellular functions. Moreover, a comprehensive description of the stable and transient protein-protein interactions (PPIs) within a cell would facilitate the functional annotation of all gene products, and provide insight into the higher-order organisation of the proteome [12,17]. Several methodologies have been developed to detect PPIs, and have been adapted to chart interactions at the proteome-wide scale. These methods, combining different technologies, experiments and computational analyses, generate PPI networks of sufficient reliability, enabling the assignment of several proteins to functional categories[31,44].

Moreover, the statistical study of bacterial PPIs over several species (meta-interactomes) has brought important knowledge about protein functions and cellular processes [39,8]. This work contributes to this line of research. Our aim is to shed light on the relationship between conservation, essentiality and functional annotation at the genetic level with connectivity patterns of PPI networks. We extend here our previous observations made on the PPI of *E.coli* which suggested a strong correlation between codon bias and the topology of PPI networks on the one hand, and between codon bias and gene conservation and essentiality on the other hand [11,10]. It is worth, in the next two paragraphs, to make more precise what is usually meant by gene essentiality and gene conservation. Individual genes in the genome contribute differentially to the survival of an organism. According to their known functional profiles and based on experimental evidence, genes can be divided into two categories: essential and nonessential ones[16,13]. Essential genes are not dispensable for the survival of an organism in the environment it lives in[13,33]. Nonessential genes are instead those which are dispensable [28], being related to functions that can be silenced without compromising the survival of the organism. Naturally, each species has adapted to one or more evolving environments and, plausibly, genes that are essential for one species may be not essential for another one.

It has been argued many times that essential genes are more conserved than nonessential ones [20,25,29,22,1]. The term "conservation" has, however, at least two meanings. On the one hand, a gene is conserved if orthologous copies of it are found in the genomes of many species, as measured by the Evolutionary Retention Index (ERI) [16,5]. On the other hand, a gene is (evolutionarily) conserved when it is subject to a purifying, selective, evolutionary pressure, which disfavors mutations. This pressure can be measured, as usually it is, by K_a/K_s , the ratio of the number of non synonymous substitutions per non synonymous site to the number of synonymous substitutions per synonymous site. To measure the evolutionary pressures exerted either on low, intermediate and high connectivity proteins we use here both K_a/K_s and the widely used ENC plots. In the second meaning a conserved gene is, in a nutshell, a slowly evolving gene [20,19].

In this work we show that bacterial PPI networks display an interesting topological-functional transition, ruled by protein connectivity k and with a threshold between $k = 40$ and $k = 50$. Proteins with high PPI network connectivities (hubs) likely correspond to genes that are conserved and essential. Conversely, genes that correspond to hub proteins in the PPI network are likely to be essential, highly shared and subject to a more purifying evolutionary pressure, than the genes coding for proteins with a low k . Additionally, below the threshold the functional repertoire of proteins is heterogeneous, whereas, above the threshold there is a quite strict functional specialization.

Materials and Methods

We consider a set of 42 bacterial genomes (that we have previously investigated in [10]), reported in Table 1. These genomes were chosen in order to have a reasonably large coverage of data concerning conservation, essentiality and selective pressure. To check the stability of the results shown in Figure 1 we have evaluated the connectivities of PPIs in an alternative set of bacterial species in Table 1. Nucleotide sequences were downloaded from the FTP server of the National Center for Biotechnology Information [4].

Gene Conservation

The Evolutionary Retention Index (ERI) [16] is a way of measuring the degree of conservation of a gene. In the present study the ERI of a gene is the fraction of genomes, among those reported in Table 1, that have at least an orthologous of the given gene. Then, as reminded in the Introduction, a low ERI value is related to a gene which is rather specific, common to a small number of genomes; whereas high ERI is characteristic of highly shared, putatively universal and essential genes.

We also make reference to another notion of gene conservation. Conserved genes are those which are subject to a purifying, conservative evolutionary pressure. To discriminate between genes subject to purifying selection and genes subject to positive selective Darwinian evolution, we use a classic but still widely used indicator, the ratio K_a/K_s between the number of non synonymous substitutions per non synonymous site (K_a) and the number of synonymous substitutions per synonymous site (K_s) [19]. Conserved genes are characterized by $K_a/K_s < 1$. We used K_a/K_s estimates by Luo [29] that are based on the method by Nej and Gojobori[32].

Gene Essentiality

We used the Database of Essential Genes (DEG, www.essentialgene.org) [29], which classifies a gene as either essential or nonessential on the basis of a combination of experimental evidence (null mutations or trasposons) and general

functional considerations. DEG collects genomes from Bacteria, Archaea and Eukarya, with different degrees of coverage[48,30]. Of the 42 bacterial genomes we consider, only 23 are covered—in toto or partially—by DEG, as indicated in Table 1.

Protein-Protein Interaction Networks

PPIs are obtained from the STRING database (Known and Predicted Protein-Protein Interactions, <https://string-db.org/>)[41]. We have chosen STRING because of its quite large coverage of different bacterial species, useful to extend to multiple species the study we did in [11]. In STRING, each interaction is assigned with a confidence level or probability w , evaluated by comparing predictions obtained by different techniques [9,34,35] with a set of reference associations, namely the functional groups of KEGG (Kyoto Encyclopedia of Genes and Genomes)[26]. In this way, interactions with high w are likely to be true positives, whereas, a low w possibly corresponds to a false positive. As usually done in the literature, we consider only interactions with $w \geq 0.9$ that allows for a fair balance between coverage and interaction reliability (see for instance the case of *E.coli* reported in [11]). We denote by k the *degree* (number of connections) associated to each proteins in each PPI network after the thresholding procedure. Note also that after applying the cut-off we are left, for each network, with a number of isolated proteins (with no connections) that grows as $\sqrt{n}$ (where n is the number of proteins in the genome). These proteins are not considered in the network analysis and are regarded as stemming from statistical noise. In order to check the robustness of the bump in Figure 1 we have also considered the degree distributions relaxing the cut-off values to $w = 0.0, 0.5$ and 0.7 ; see Figures 1, 2 and 3 of Supplementary Information.

K_a/K_s

K_a/K_s is the ratio of nonsynonymous substitutions per nonsynonymous site (K_a) to the number of synonymous substitutions per synonymous site (K_s) [19]. This parameter is widely accepted as a straightforward and effective way of separating genes subject to purifying evolutionary selection ($K_a/K_s < 1$) from genes subject to positive selective Darwinian evolution ($K_a/K_s > 1$). There are different methods to evaluate this ratio, though the alternative approaches are quite consistent among themselves. For the sake of comparison, we have used here the K_a/K_s estimates by Luo et al. [29] which are based on the Nej and Gojobori method [32]. Note that each genome has a specific average level of K_a/K_s . In Figure 3 average values of K_a/K_s are shown for low, intermediate, high connectivity bins of genes.

ENC plot

The ENC-plot is a well known tool to investigate the patterns of synonymous codon usage in which the *ENC* values are plotted against GC_3 values when codon usage is dominated by the mutational bias, the formula of expected *ENC* values is given by:

$$ENC = 2 + s + \frac{29}{s^2 + (1 - s)^2} \quad (1)$$

s represents the value of GC_3 [46]. When the corresponding points fall near the expected neutral curve, mutations that enforce the typical mutational bias of the species are the main factor affecting the observed codon diversity. Whereas when the corresponding points fall considerably below the expected curve, the observed CUB is mainly affected by natural selection. To quantitatively represent the balance between mutational bias and selective natural pressure we parametrize the ENC formula, to be used in non-linear fits to the experimental data:

$$ENC = a + b * s + \frac{c}{s^2 + d * (1 - s)^2} \quad (2)$$

Clusters of orthologous proteins

We use the functional annotation given in the database of orthologous groups of proteins (COGs) from Koonin's group, available at <http://ncbi.nlm.nih.gov/COG/> [42, 15]. We consider 15 functional COG categories (see Table 2), excluding the generic categories R and S for which functional annotation is too general or missing.

Results and discussion

Degree distribution of PPI networks. We start by studying the degree distributions $P(k)$ observed in bacterial PPIs. We first recall that such a distribution was found to be scale-free in *E.coli* [7, 24, 36, 11], meaning that the corresponding PPI network features a large number of poorly connected proteins, and a relatively small number of highly connected hubs. In order to assess the generality of this observation, we compute $P(k)$ for each genome in Table 1 (plots are reported in Figures S4-S5 of the Supplementary Information). Note that, despite that PPIs of different bacteria have different sizes and densities, their average connectivity and the support of their $P(k)$ are very similar (as shown in Figure S1 of the Supplementary Information). Thus, we can superpose all the considered bacterial degree distributions without the need to normalise the support of each $P(k)$. When doing so, we observe two distinct regimes (see

Figure 1). For high values of k , the distribution is scale-free: $P(k) \propto k^{-\gamma}$. This scaling behaviour is consistent with previous studies on the genomes of yeast, worms and flies [18] and on co-conserved PPIs in some bacteria [27]. For higher values of k , however, the distribution deviates from a power law, and a bump with a Gaussian-like shape emerges. Interestingly, this feature is almost undetectable in the graph relative to a single species (such as, e.g., *E. coli*, see Figure 2 in Supplementary Information of Dilucca et al. [11]), but clearly emerges when the statistics is enriched by adding together the graphs relative to several species. This feature, emerging for $k \geq 40$ is reasonably due to the contribution of proteins belonging to complexes [47]. In particular, if one recalculates the degree distribution of a data set in which the ribosomal proteins are removed the bump is not present (see figure1, empty dots). Moreover, if we consider the separate contribution of essential and nonessential genes to the $P(k)$ (for DEG-annotated genomes), we see that the superposed peak is present only in the degree distribution of essential genes. Moreover, the degree distributions for essential and nonessential genes are well separated and the average degree is systematically higher for essential genes than for nonessential ones—consistently with previous findings [18].

Fig. 1 Probability distribution $P(k)$ for the number of connections k of each protein, averaged over the bacterial species considered in Table 1 (full dots), compared with the degree distribution after removal of the proteins corresponding to genes in COG J, related to translational processes (empty dots). Inset: $P(k)$ for essential (E) and nonessential (NE) genes, averaged over DEG-annotated genomes. Note that the average degree is higher for essential genes than for nonessential ones, and the two probability distributions are quite distinct. The region of the curve for low k can be well approximated by a power law [2].

A critical remark is in order at this point. One could ask how many species are needed to obtain the bump observed in figure 1 as a stable feature? To check this point we have evaluated the degree distributions obtained by gradually averaging over an increasing number of species, taken in an arbitrary order.

As shown in figure S2 it is sufficient to average the $P(k)$ over no more than 10 species to have the bump consistently emerge and stabilise as a self-averaging feature, that is mainly associated to the complex of ribosomal proteins.

Moreover, we have checked that the observed bump is still present averaging the degree distributions in the PPIs of 42 alternative bacterial species (see figure S3). Then, we can conclude that it is sufficient to average over just a few species to let the the bump emerge as a general feature.

PPI connectivity and gene conservation We now investigate whether the connectivity k of a protein in a PPI network drives a transition in the degree of conservation (as measured by ERI) of the corresponding genes. Figure 2 displays the average value and the spread of ERI in genes relative to bins of proteins that are iso-connected in the PPIs of different species. As a general feature we observe that, on the average, the genes of highly connected proteins are highly conserved among the bacterial species we consider, that constitute a reasonably wide sample of different evolutionary adaptations. The same figure 2 shows that if $k \leq 50$ then the ERI highly fluctuates between different samples of proteins with the same k , in different species. For high connectivities (above $k = 50$), the ERI is close to 1, with a drastic drop in the fluctuation (as shown in the inset). This observation points to the existence, in each bacterial PPI, of an almost-invariant structure of conserved hubs, sustained by highly conserved genes. We have checked that a connectivity of 40 acts as a lower bound, then we can conclude, as a rule of thumb, that a protein with connectivity degree of 40 or more is likely to be coded by a gene shared by at least 80% of the species in a generic pool of bacteria. At the moment is hard to figure out deep and general biophysical facts that could give a universal character to the threshold we are observing here, in the form of a new biological law. Let us just propose, as an heuristic observation, the existence of a characteristic, almost-critical value of connectivity to be set close to 50.

Evolutionary pressure and PPI connectivity We then look at the evolutionary pressure exerted on genes whose proteins have different connectivities. The graph in figure 3 shows the ratio K_a/K_s for groups of genes binned by the connectivity k of the corresponding proteins, for all the 42 bacterial species in table 1. As is well known this ratio K_a/K_s provides a straightforward indication of the balance between a positive driving *darwinian selection* (when the numerator prevails) and a *purifying*, stabilizing selection (acting against change in genes for which the denominator prevails).

We see that the more connected proteins correspond to genes which are subject to an increasing purifying evolutionary pressure. Indeed, (K_a/K_s) is less than 1 in all bins of connectivity and systematically decreases, as a function of k , down to zero. A decreasing ratio generally indicates an increasing role of the purifying conservative selection in the corresponding set of genes.

To add evidence to this observation we have also considered ENC plots for sets of genes binned by the connectivities of the corresponding proteins. Interestingly, the data in figure 4 are fully consistent with those in figure 3. In

Fig. 2 Average ERI values of bacterial genes as a function of the degrees k of the corresponding proteins, for all the considered genomes. Error bars are standard deviations of ERI values associated to a given k value. Inset: amplitude of the error bar (ΔERI) as a function of k .

Fig. 3 Dependence of purifying selective pressure (K_a/K_s) in groups of genes corresponding to proteins with different connectivity degrees k .

the ENC plots, the points associated to low connectivity proteins (green) are closer to the so called Wright's profiles (represented as black solid lines) than those associated to proteins with intermediate and high connectivities (red and blue lines). Figure 5 stresses this observation in a more quantitative way by showing that in the ENC plots the average distance from Wright's profile monotonously increases with k . Overall, the above results clearly indicate that codon bias and GC content of high connectivity genes are more under selective darwinian pressure than genes coding for low-connectivity proteins, in which the rate of accepted mutations is mainly ruled by mutational bias.

Fig. 4 ENC plots for three groups of genes corresponding to proteins with different degree connectivities. In each panel the solid black lines are plots of Wright's theoretical curve (equation 1) which correlates effective number of codons with GC_3 in the case of pure mutational bias (no selective pressure). Coherently with figure 3 the case of low connectivities are shown in red, intermediate in green and high connectivities in blue. In the bottom-right panel dashed linear non-linear fits of Wright's theoretical shapes to the experimental data. The fitted curves are shown together in the bottom right panel. For the sake of completeness the best fit parameters are reported in table 5.

Fig. 5 Average distance d from Wright's curve as a function of k . The distance from the curve increases with k .

PPI and Essentiality. To further investigate the relationship between gene essentiality and protein connectivities, we consider DEG-annotated genomes and classify interactions between proteins (links) making references to the essentiality of the corresponding genes. We distinguish three sets of links: ee (linking proteins from two essential genes), $\bar{e}\bar{e}$ (from two nonessential genes)

and $e\bar{e}$ (from an essential gene and a nonessential one). We then compute the *density* of these sets of links respectively as:

$$\rho_{ee} = \frac{|ee|}{\frac{1}{2}E(E-1)}, \quad \rho_{\bar{e}\bar{e}} = \frac{|\bar{e}\bar{e}|}{\frac{1}{2}NE(NE-1)}, \quad \rho_{e\bar{e}} = \frac{|e\bar{e}|}{\frac{1}{2}E \cdot NE}, \quad (3)$$

where E and NE denote the number of essential and nonessential genes, respectively (self-connections are excluded in our analysis). Such densities are then compared with the overall density of the network—restricted to genes classified as either essential or nonessential:

$$\langle \rho \rangle = \frac{|ee| + |\bar{e}\bar{e}| + |e\bar{e}|}{\frac{1}{2}(E + NE)(E + NE - 1)}. \quad (4)$$

We use the ratios $r_{ee} = \rho_{ee}/\langle \rho \rangle$, $r_{\bar{e}\bar{e}} = \rho_{\bar{e}\bar{e}}/\langle \rho \rangle$ and $r_{e\bar{e}} = \rho_{e\bar{e}}/\langle \rho \rangle$ to assess the level of connectivity of the subnetworks with respect to the overall connectivity. Table 3 shows that subnetworks of essential genes are far denser than the overall networks, and that, in general, essential and nonessential genes tend to form network components that are weakly interconnected. This happens because many essential genes encode for ribosomal proteins, which in turn are localized in the ribosome so that they have a major probability to interact [3]. Figures S4-S5 of the Supplementary Information display such network features for each individual species.

PPI connectivity and functional specialization. For each PPI network, we define the conditional probability that a protein with degree k belongs to a given COG as:

$$P(\text{COG}|k) = P(k|\text{COG})P(\text{COG})/P(k), \quad (5)$$

where $P(k)$ is the degree distribution in the PPI network, $P(\text{COG})$ is the frequency of that COG in the proteome, and $P(k|\text{COG})$ is the degree distribution restricted to that COGs. Figure 6 shows the COG spectrum as a function of k over all bacteria species considered. Interestingly, we again note a marked transition. Below $k \simeq 40$ the COG spectrum is quite heterogeneous: genes corresponding to proteins with low connectivity are spread over several COGs which correspond to different functions (see Table 2).

The transition shows that proteins with more than 40 interactions are likely to be coded by genes belonging to COG J, which encompasses translation processes and ribosomal functions. There are yet a handful of outliers, hubs with connectivities between 57 and 62, that belong to COG I (related to lipid transport and metabolism) and K and L (which, together with J, define the functional class of information storage and processing). The list of these outliers is reported in Table 4. But, which are the genes of COG J that drive the transition? In the next Figures 7 and 8 we are able to show which genes are the main characters in the transition. We investigated then the connectivities of the highly conserved (shared by all the species in Table1) genes belonging to COG J and whose proteins have connectivities bigger than 40. These highly

shared genes corresponding to cores of highly connected ribosomal proteins are listed in Table 6. In the heatmap of Figure 7 we sort each gene in the COG J in order of ascending degree, species by species, and we see there is a core of genes (in red, lower left sector) that correspond to highly connected proteins, which are also highly shared (ERI=1, see Table 6) among all the species we considered. It is quite clear that in the heat map of Figure 7 the 42 species in this study can be splitted into at least four groups. Each group of species is characterized by different connectivities of their conserved ribosomal proteins. In other words, this observation suggests that the abrupt transition shown in Figure 6 is driven by a subset of COG J genes which are listed in Table 6 corresponds detailed connectivity of highly conserved ribosomal proteins. In order to show how conservation of the gene and connectivity of the protein are correlated we have built the connectivity map of the proteins corresponding to the COG J genes (Figure 8). The map in Figure 8 shows in a color code how much is conserved a given interaction (e.g., the red color corresponds to links in the PPI that are conserved among 37 species over 42). It is evident in this map that the set of highly conserved COG J genes of Table 6 corresponds to the core of highly connected ribosomal proteins, which are approximately invariant among several species.

Fig. 6 Probability distribution $P(\text{COG}|k)$ of belonging to a given COG for proteins with degree k , over all considered genomes. Proteins with low connectivity have a very heterogeneous COG composition, whereas, those with high k basically belong only to COG J.

Conclusions

Topological analysis of biological networks, such as protein-protein interaction or metabolic networks, has demonstrated that structural features of network subgraphs are correlated with biological functions [40,37]. For instance, it was shown that highly connected patterns of proteins in a PPI are fundamental to cell viability [23]. In this work we have shown the existence of a topological-functional transition in bacterial species, ruled by the connectivity of proteins

Fig. 7 Heat map of degree of genes class COG J for each species. Genes are sorted by averaging degree. We note that genes with average degree major than 40 are conserved for all species. Details of these genes are in Table 6.

in the PPI networks. The threshold in k of the transition is located between $k = 40$ and $k = 50$. Proteins that have connectivities above the threshold are mostly encoded by genes that are conserved (as measured both by ERI and K_a/K_s) and essential. Moreover the functional repertoire above the threshold focuses mainly on the COG J (Translation, ribosomal structure and biogenesis), with just a few interesting hubs belonging to COGs I (Lipid transport and metabolism), K (Transcription) and L (Replication, recombination and repair).

Indeed, the PPI network of each bacterial species is characterized by a highly connected core of conserved ribosomal proteins, the components of multi-subunit complexes whose corresponding genes are mostly essential [7, 27] and code for supra-molecular complexes, that pile up in the bump we have observed for the degree distribution (figure1). Hence, what we are seeing here is essentially the ribosome, and related protein complexes such as RNA Polymerase. Indeed, the ribosome is the only molecular machine in bacteria in which a given protein could legitimately have 40 or more protein binding partners, with the help of rRNA mediating interactions [14].

We believe that the observations we have presented here can have implications both for the prediction of gene essentiality, based on the knowledge of PPI networks, and for the prediction of interactions between proteins, based on genetic information[21,45]. It is interesting to note that our results are consistent with a previous study based on inferred bacterial co-conserved networks based on phylogenetic profiles [27]. The coupled flows of information

Fig. 8 Connectivity map of the proteins coded by COG J genes. The color code measures the degree of conservation of interactions between proteins among the various species. When the link is present in all bacteria the number of conservation is 42, whereas when the link is not present the number is 0.

from the genetic level up to the proteomic level and vice-versa should be further systematically investigated, taking into account archaeal and prokaryotic genomes in the search for emerging multi-layer structures that could offer basic theoretical grounds for clinical and systemic applications, for instance related to antimicrobial resistances [43,49,38,6].

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Alvarez-Ponce, D., Sabater-Muñoz, B., Toft, C., Ruiz-González, M.X., Fares, M.A.: Essentiality is a strong determinant of protein rates of evolution during mutation accumulation experiments in *escherichia coli*. *Genome Biology and Evolution* **8**(9), 2914–2927 (2016). DOI 10.1093/gbe/evw205
2. Annibale, A., Coolen, A.C., Planell-Morell, N.: Protein interaction networks and biology: Towards the connection. *arXiv:1501.00662* (2015)
3. Bader, G.D., Hogue, C.W.V.: An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinformatics* **4**(2) (2003). DOI 10.1186/1471-2105-4-2
4. Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W.: Genbank. *Nucleic Acids Research* **41**(D1), D36–D42 (2013). DOI 10.1093/nar/gks1195
5. Bergmiller, T., Ackermann, M., Silander, O.K.: Patterns of evolutionary conservation of essential genes correlate with their compensability. *PLoS Genetics* **8**(6), e1002803 (2012). DOI 10.1371/journal.pgen.1002803
6. Boccaletti, S., Bianconi, G., Criado, R., del Genio, C., nes, J.G.G., Romance, M., na Nadal, I.S., Wang, Z., Zanin, M.: The structure and dynamics of multilayer networks. *Physics Reports* **544**(1), 1–122 (2014). DOI 10.1016/j.physrep.2014.07.001
7. Butland, G., Peregrin-Alvarez, J.M., Li, J., Yang, W., Yang, X., Canadien, V., Starostine, A., Richards, D., Beattie, B., Krogan, N., Davey, M., Parkinson, J., Greenblatt, J., Emili, A.: Interaction network containing conserved and essential protein complexes in *escherichia coli*. *Nature* **433**, 531–537 (2005). DOI 10.1038/nature03239
8. Caufield, J.H., Wimble, C., Shary, S., Wuchty, S., Uetz, P.: Bacterial protein meta-interactomes predict cross-species interactions and protein function. *BMC Bioinformatics* **18**(1), 171 (2017). DOI 10.1186/s12859-017-1585-0
9. Chien, C.T., Bartel, P.L., Sternglanz, R., Fields, S.: The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proceedings of the National Academy of Science* **88**(1), 9578–9582 (1991). DOI 10.1073/pnas.88.21.9578
10. Dilucca, M., Cimini, G., Giansanti, A.: Essentiality, conservation, evolutionary pressure and codon bias in bacterial genomes. *Gene* **663**, 178 – 188 (2018). DOI <https://doi.org/10.1016/j.gene.2018.04.017>. URL <http://www.sciencedirect.com/science/article/pii/S0378111918303858>
11. Dilucca, M., Cimini, G., Semmoloni, A., Deiana, A., Giansanti, A.: Codon bias patterns of *e. coli*'s interacting proteins. *PLoS ONE* **10**(11), e0142127 (2015). DOI 10.1371/journal.pone.0142127
12. Drewes, G., Bouwmeester, T.: Global approaches to protein–protein interactions. *Current Opinion in Cell Biology* **15**(2), 199–205 (2003). DOI 10.1016/S0955-0674(03)00005-X
13. Fang, G., Rocha, E., Danchin, A.: How essential are nonessential genes? *Molecular Biology and Evolution* **22**(11), 2147–2156 (2005). DOI 10.1093/molbev/msi211
14. Fox, G.E.: Origin and evolution of the ribosome. *Cold Spring Harbor Perspectives in Biology* **2**(9) (2010). DOI 10.1101/cshperspect.a003483
15. Galperin, M.Y., Makarova, K.S., Wolf, Y.I., Koonin, E.V.: Expanded microbial genome coverage and improved protein family annotation in the cog database. *Nucleic Acids Research* **43**(D1), D261 (2015). DOI 10.1093/nar/gku1223
16. Gerdes, S., Scholle, M., Campbell, J., Balazsi, G., Ravasz, E., Daugherty, M., Somera, A., Kyrpides, N., Anderson, I., Gelfand, M., et al.: Experimental determination and system level analysis of essential genes in *escherichia coli* mg1655. *Journal of Bacteriology* **185**(19), 5673–5684 (2003). DOI 10.1128/JB.185.19.5673-5684.2003
17. Golemis, E., Adams, P.: Protein-protein Interactions: A Molecular Cloning Manual. Cold Spring Harbor Laboratory Press (2005)
18. Hahn, M.W., Kern, A.D.: Comparative genomics of centrality and essentiality in three eukaryotic protein-interaction networks. *Molecular Biology and Evolution* **22**(4), 803–806 (2005). DOI 10.1093/molbev/msi072

19. Hurst, L.D.: The K_a/K_s ratio: diagnosing the form of sequence evolution. *Trends in Genetics* **18**, 486–487 (2002). DOI 10.1016/S0168-9525(02)02722-1
20. Hurst, L.D., Smith, N.G.: Do essential genes evolve slowly? *Current Biology* **9**(14), 747–750 (1999). DOI 10.1016/S0960-9822(99)80334-0
21. Hwang, Y.C., Lin, C.C., Chang, J.Y., Mori, H., Juan, H.F., Huang, H.C.: Predicting essential genes based on network and sequence analysis. *Molecular BioSystems* **5**, 1672–1678 (2009). DOI 10.1039/B900611G
22. Ish-Am, O., Kristensen, D.M., Rupp, E.: Evolutionary conservation of bacterial essential metabolic genes across all bacterial culture media. *PLoS ONE* **10**(4), e0123785 (2015). DOI 10.1371/journal.pone.0123785
23. Jeong, H., Mason, S.P., Barabasi, A.L., Oltvai, Z.N.: Lethality and centrality in protein networks. *Nature* **411**(6833), 41–42 (2001). DOI 10.1038/35075138
24. Jin, Y., Turaev, D., Weinmaier, T., Rattei, T., Makse, H.A.: The evolutionary dynamics of protein-protein interaction networks inferred from the reconstruction of ancient networks. *PLoS ONE* **8**, e58134 (2013). DOI 10.1371/journal.pone.0058134
25. Jordan, I.K., Rogozin, I.B., Wolf, Y.I., Koonin, E.V.: Essential genes are more evolutionarily conserved than are nonessential genes in bacteria. *Genome Research* **12**(6), 962–968 (2002). DOI 10.1101/gr.87702
26. Kanehisa, M., Goto, S.: Kegg: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* **28**(1), 27 (2000). DOI 10.1093/nar/28.1.27
27. Karimpour-Fard, A., Leach, S.M., Hunter, L.E., Gill, R.T.: The topology of the bacterial co-conserved protein network and its implications for predicting protein function. *BMC Genomics* **9**(1), 313 (2008). DOI 10.1186/1471-2164-9-313
28. Lin, Y., Gao, F., Zhang, C.T.: Functionality of essential genes drives gene strand-bias in bacterial genomes. *Biochemical and Biophysical Research Communications* **396**(2), 472–476 (2010). DOI 10.1016/j.bbrc.2010.04.119
29. Luo, H., Gao, F., Lin, Y.: Evolutionary conservation analysis between the essential and nonessential genes in bacterial genomes. *Scientific Reports* **5**, 13210 (2015). DOI 10.1038/srep13210
30. Luo, H., Lin, Y., Gao, F., Zhang, C.T., Zhang, R.: Deg 10, an update of the database of essential genes that includes both protein-coding genes and noncoding genomic elements. *Nucleic Acids Research* **42**(D1), D574–D580 (2014). DOI 10.1093/nar/gkt1131
31. von Mering, C., Krause, R., Snel, B., Cornell, M., Oliver, S.G., Fields, S., Bork, P.: Comparative assessment of large-scale data sets of protein-protein interactions. *Nature* **417**, 399–403 (2002). DOI 10.1038/nature750
32. Nei, M., Gojobori, T.: Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Molecular Biology and Evolution* **3**(5), 418 (1986). DOI 10.1093/oxfordjournals.molbev.a040410
33. Peng, C., Gao, F.: Protein localization analysis of essential genes in prokaryotes. *Scientific Reports* **4**, 6001 (2014). DOI 10.1038/srep06001
34. Phizicky, E.M., Fields, S.: Protein-protein interactions: Methods for detection and analysis. *Microbiological Reviews* **59**(1), 94–123 (1995)
35. Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., Séraphin, B.: The tandem affinity purification (tap) method: A general procedure of protein complex purification. *Methods* **24**(3), 218–229 (2001). DOI 10.1006/meth.2001.1183
36. Rajagopala, S.V., Sikorski, P., Kumar, A., Mosca, R., Vlasblom, J., Arnold, R., Franca-Koh, J., Pakala, S.B., Phanse, S., Ceol, A., Häuser, R., Siszler, G., Wuchty, S., Emili, A., Babu, M., Aloy, P., Pieper, R., Uetz, P.: The binary protein-protein interaction landscape of escherichia coli. *Nature Biotechnology* **32**, 285–290 (2014). DOI 10.1038/nbt.2831
37. Rao, V.S., Srinivas, K., Sujini, G.N., Kumar, G.N.S.: Protein-protein interaction detection: methods and analysis. *International Journal of Proteomics* **2014**(147648) (2014). DOI 10.1155/2014/147648
38. Sevimoglu, T., Arga, K.Y.: The role of protein interaction networks in systems biomedicine. *Computational and Structural Biotechnology Journal* **11**(18), 22–27 (2014). DOI 10.1016/j.csbj.2014.08.008

39. Shatsky, M., Allen, S., Gold, B.L., Liu, N.L., Juba, T.R., Reveco, S.A., Elias, D.A., Prathapam, R., He, J., Yang, W., Szakal, E.D., Liu, H., Singer, M.E., Geller, J.T., Lam, B.R., Saini, A., Trotter, V.V., Hall, S.C., Fisher, S.J., Brenner, S.E., Chhabra, S.R., Hazen, T.C., Wall, J.D., Witkowska, H.E., Biggin, M.D., Chandonia, J.M., Butland, G.: Bacterial interactomes: Interacting protein partners share similar function and are validated in independent assays more frequently than previously reported. *Mol Cell Proteomics* **15**(5), 1539–55 (2016). DOI 10.1074/mcp.M115.054692
40. Srinivasan, B.S., Novak, A.F., Flannick, J.A., Batzoglou, S., McAdams, H.H.: Integrated Protein Interaction Networks for 11 Microbes, pp. 1–14. Springer Berlin Heidelberg (2006). DOI 10.1007/117329901
41. Szklarczyk, M.J.: The string database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Research* **45**(Database issue), D362–D368 (2017). DOI 10.1093/nar/gkq973
42. Tatusov, R.L., Natale, D.A., Garkavtsev, I.V., Tatusova, T.A., Shankavaram, U.T., Rao, B.S., Kiryutin, B., Galperin, M.Y., Fedorova, N.D., Koonin, E.V.: The cog database: New developments in phylogenetic classification of proteins from complete genomes. *Nucleic Acids Research* **29**(1), 22 (2001). DOI 10.1093/nar/29.1.22
43. Terradot, L., Noirot-Gros, M.F.: Bacterial protein interaction networks: puzzle stones from solved complex structures add to a clearer picture. *Integrative Biology* **3**, 645–652 (2011). DOI 10.1039/C0IB00023J
44. Tong, A.H.Y., Drees, B., Nardelli, G., Bader, G.D., Brannetti, B., Castagnoli, L., Evangelista, M., Ferracuti, S., Nelson, B., Paoluzi, S., Quondam, M., Zucconi, A., Hogue, C.W.V., Fields, S., Boone, C., Cesareni, G.: A combined experimental and computational strategy to define protein interaction networks for peptide recognition modules. *Science* **295**(5553), 321–324 (2002). DOI 10.1126/science.1064987
45. Wei, W., Ning, L.W., Ye, Y.N., Guo, F.B.: Geptop: A gene essentiality prediction tool for sequenced bacterial genomes based on orthology and phylogeny. *PLoS ONE* **8**(8), e72343 (2013). DOI 10.1371/journal.pone.0072343
46. Wright, F.: The “effective number of codons” used in a gene. *Gene* **87**(1), 23–29 (1990). DOI 10.1016/0378-1119(90)90491-9
47. Wuchty, S., Uetz, P.: Protein-protein interaction networks of *E. coli* and *S. cerevisiae* are similar. *Scientific Reports* **4**, 7187 (2014). DOI 10.1038/srep07187
48. Zhang, R., Lin, Y.: Deg 5.0, a database of essential genes in both prokaryotes and eukaryotes. *Nucleic Acids Research* **37**(suppl 1), D455–D458 (2009)
49. Zoraghi, R., Reiner, N.E.: Protein interaction networks as starting points to identify novel antimicrobial drug targets. *Curr Opin Microbiol* **16**(5), 566–72 (2013). DOI 10.1016/j.mib.2013.07.010

Organisms	Abbr.	Class	RefSeq	STRING	n
<i>Agrobacterium tumefaciens</i> (fabrum)	agtu	1	NC_003062	176299	2765
<i>Aquifex aeolicus</i> VF5	aqae	12	NC_000918	224324	1497
<i>Bacillus subtilis</i> 168	basu	6	NC_000964	224308	4175
<i>Bacteroides thetaiotaomicron</i> VPI-5482	bath	7	NC_004663	226186	4778
<i>Brucella melitensis</i> bv. 1 str. 16M	brme	1	NC_003317.1	224914	2059
<i>Burkholderia pseudomallei</i> K96243	bups	3	NC_006350	272560	3398
<i>Buchnera aphidicola</i> Sg uid57913	busg	2	NC_004061	198804	546
<i>Burkholderia thailandensis</i> E264	buth	2	NC_007651	271848	3276
<i>Caulobacter crescentus</i>	cacr	1	NC_011916	565050	3885
<i>Campylobacter jejuni</i>	caje	4	NC_002163	192222	1572
<i>Chlamydia trachomatis</i> D/UW-3/CX	chtr	14	NC_000117.1	272561	894
<i>Clostridium acetobutylicum</i> ATCC 824	clac	8	NC_003030.1	272562	3602
<i>Deinococcus radiodurans</i> R1	dera	9	NC_001263.1	243230	2629
<i>Escherichia coli</i> K-12 MG1655	esco	3	NC_000913.3	511145	4004
<i>Francisella novicida</i> U112	frno	3	NC_008601	401614	1719
<i>Fusobacterium nucleatum</i> ATCC 25586	funu	15	NC_003454.1	190304	1983
<i>Haemophilus influenzae</i> Rd KW20	hain	3	NC_000907.1	71421	1610
<i>Helicobacter pylori</i> 26695	hepy	4	NC_000915	85962	1469
<i>Listeria monocytogenes</i> EGD-e	limo	6	NC_003210.1	169963	2867
<i>Mesorhizobium loti</i> MAFF303099	melo	1	NC_002678.2	266835	6743
<i>Mycoplasma genitalium</i> G37	myge	10	NC_000908	243273	475
<i>Mycoplasma pneumoniae</i> M129	mypn	10	NC_000912.1	272634	648
<i>Mycoplasma pulmonis</i> UAB CTIP	mypu	10	NC_002771	272635	782
<i>Mycobacterium tuberculosis</i> H37Rv	mytu	5	NC_000962.3	83332	3936
<i>Neisseria gonorrhoeae</i> FA 1090 uid57611	nego	2	NC_002946	242231	1894
<i>Porphyromonas gingivalis</i> ATCC 33277	pogi	7	NC_010729	431947	2089
<i>Pseudomonas aeruginosa</i> UCBPP-PA14	psae	3	NC_008463	208963	5892
<i>Ralstonia solanacearum</i> GMI1000	raso	2	NC_003295.1	267608	3436
<i>Rickettsia prowazekii</i> str. Madrid E	ripr	1	NC_000963.1	272947	8433
<i>Salmonella enterica</i> serovar Typhi	saen	3	NC_004631	209261	4352
<i>Shewanella oneidensis</i> MR-1	shon	3	NC_004347	211586	4065
<i>Sinorhizobium meliloti</i> 1021	sime	1	NC_003047.1	266834	3359
<i>Sphingomonas wittichii</i> RW1	spwi	1	NC_009511	392499	4850
<i>Staphylococcus aureus</i> N315	stau	6	NC_002745.2	158879	2582
<i>Staphylococcus aureus</i> NCTC 8325	stau_	6	NC_007795	93061	2767
<i>Streptococcus pyogenes</i> NZ131	stpy	6	NC_011375	471876	1700
<i>Streptococcus sanguinis</i>	stsa	6	NC_009009	388919	2270
<i>Synechocystis</i> sp. PCC 6803	syp	13	NC_000911.1	1148	3179
<i>Thermotoga maritima</i> MSB8	thma	16	NC_000853.1	243274	1858
<i>Treponema pallidum</i> Nichols	trpa	11	NC_000919.1	243276	1036
<i>Vibrio cholerae</i> N16961	vich	3	NC_002505	243277	2534
<i>Xylella fastidiosa</i> 9a5c	xyfa	3	NC_002488	160492	2766

Table 1 Summary of the selected bacterial dataset. Organism name, abbreviation, class, RefSeq, STRING code, size of genome (number of genes *n*). Genomes annotated in the Database of Essential Genes (DEG) are highlighted with bold fonts. Classes are: Alphaproteobacteria(1), Betaproteobacteria(2), Gammaproteobacteria(3), Epsilonproteobacteria(4), Actinobacteria(5), Bacilli(6), Bacteroidetes(7), Firmicutes(8), Fusobacteria(9), Planctomycetes(10), Proteobacteria(11), Spirochaetes(12), Thaumarchaeota(13), Thermotoga(14), Verrucomicrobia(15), and other(16).

COG ID	Functional classification
	<i>INFORMATION STORAGE AND PROCESSING</i>
J	Translation, ribosomal structure and biogenesis
K	Transcription
L	Replication, recombination and repair
	<i>CELLULAR PROCESSES AND SIGNALING</i>
D	Cell cycle control, cell division, chromosome partitioning
T	Signal transduction mechanisms
M	Cell wall/membrane/envelope biogenesis
N	Cell motility
O	Post-translational modification, protein turnover, chaperones
	<i>METABOLISM</i>
C	Energy production and conversion
G	Carbohydrate transport and metabolism
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
H	Coenzyme transport and metabolism
I	Lipid transport and metabolism
P	Inorganic ion transport and metabolism

Table 2 Functional classification of COG clusters.

Organisms	r_{ee}	$r_{\bar{e}\bar{e}}$	$r_{e\bar{e}}$
basu	44.46	0.80	0.11
bath	20.07	0.76	0.25
bups	6.21	0.83	0.27
buth	18.69	0.70	0.22
cacr	18.40	0.70	0.15
caje	3.65	0.82	0.32
esco	2.91	0.88	0.31
frno	9.84	0.52	0.18
hain	1.65	1.15	0.27
hepy	2.91	0.78	0.38
myge	1.42	0.29	0.08
mypu	3.42	0.22	0.12
mytu	8.09	0.78	0.23
pogi	11.03	0.41	0.21
psae	9.85	0.92	0.16
saen	28.80	0.81	0.12
shon	6.50	0.64	0.16
spwi	15.47	0.74	0.22
stau	23.05	0.58	0.23
stau_	21.89	0.64	0.16
stpy	9.30	0.73	0.23
stsa	30.65	0.61	0.22
vich	8.37	0.81	0.19

Table 3 Relative density values r for PPI subnetworks between essential genes (r_{ee}), between nonessential genes ($r_{\bar{e}\bar{e}}$) and between essential and nonessential genes ($r_{e\bar{e}}$), for each DEG-annotated bacterial genome.

<i>k</i>	COG	Gene	Protein
57	1250I	paaH	3-hydroxyadipyl-CoA dehydrogenase, NADdependent
	0365I	acs	acetyl-CoA synthetase
	1250I	paaH	3-hydroxyadipyl-CoA dehydrogenase, NADdependent
58	0222J	rplL	50S ribosomal subunit protein L7/L12
	0335J	rplS	50S ribosomal subunit protein L19
	0267J	rpmG	50S ribosomal subunit protein L33
	0365I	acs	acetyl-CoA synthetase
59	0183I	paaJ	3-oxoadipyl-CoA3-oxo-5,6-dehydrosuberil-CoA thiolase
	1960I	ydiO	putative acyl-CoA dehydrogenase
	0183I	atoB	acetyl-CoA acetyltransferase
60	0197J	rplP	50S ribosomal subunit protein L16
	0088J	rplD	50S ribosomal subunit protein L4
	0197J	rplP	50S ribosomal subunit protein L16
	0087J	rplC	50S ribosomal subunit protein L3
	1960I	aidB	putative acyl-CoA dehydrogenase
61	0085K	rpoB	RNA polymerase, beta subunit
	0202K	rpoA	RNA polymerase, alpha subunit
62	0087J	rplC	50S ribosomal subunit protein L3
	0052J	rpsB	30S ribosomal subunit protein S2
	2965L	PriB	ribosomal replication protein

Table 4 Specific hubs. In this table we detail which proteins populate the few bins of connectivity around $k = 60$ in figure 6.

k	a	b	c	d	R^2
[0 – 10]	40,561	-10,338	5,555	1,052	0,617
[10 – 20]	23,774	3,890	8,583	0,626	0,590
[20 – 30]	20,280	8,287	8,276	0,507	0,540
[30 – 40]	18,296	10,685	8,334	0,190	0,790
[40 – 50]	18,548	10,372	8,326	0,495	0,589
[50 – 60]	25,868	2,508	8,038	0,650	0,758
[60 – 70]	29,344	-0,756	10,224	0,977	0,870
[70 – 80]	30,507	3,438	6,990	0,811	0,874

Table 5 Best fit values of the parameters in equation 1 and correlation coefficients for different connectivity data, shown in figure 4.

COG	Genes name	<k >
COG0097J	50S ribosomal protein L6	60.24
COG0087J	50S ribosomal protein L3	60.19
COG0197J	50S ribosomal protein L16	60.19
COG0090J	50S ribosomal protein L2	60.14
COG0080J	50S ribosomal protein L11	60.12
COG0088J	50S ribosomal protein L4	60.12
COG0081J	50S ribosomal protein L1	58.19
COG0089J	50S ribosomal protein L23	57.88
COG0102J	50S ribosomal protein L13	57.45
COG0094J	50S ribosomal protein L5	57.21
COG0092J	30S ribosomal protein S3	57.12
COG0098J	30S ribosomal protein S5	57.10
COG0093J	50S ribosomal protein L14	57.00
COG0091J	50S ribosomal protein L22	56.24
COG0049J	30S ribosomal protein S7	55.31
COG0051J	30S ribosomal protein S10	55.24
COG0200J	50S ribosomal protein L15	55.12
COG0256J	50S ribosomal protein L18	54.86
COG0203J	50S ribosomal protein L17	54.43
COG0244J	50S ribosomal Protein L10	54.19
COG0100J	30S ribosomal protein S11	53.76
COG0522J	30S ribosomal protein S4	53.43
COG0096J	30S ribosomal protein S8	53.10
COG0099J	30S ribosomal protein S13	52.88
COG0048J	30S ribosomal protein S12	52.14
COG0198J	50S ribosomal protein L24	50.83
COG0185J	30S ribosomal protein S19	50.52
COG0199J	30S ribosomal protein S14	50.45
COG0103J	30S ribosomal protein S9	49.45
COG0480J	tetracycline resistance protein. tetM	47.90
COG0052J	30S ribosomal protein S2	47.69
COG0184J	30S ribosomal protein S15	45.95
COG0186J	30S ribosomal protein S17	44.60
COG0255J	50S ribosomal protein L29	43.95
COG0222J	50S ribosomal protein L7/L12	42.43
COG1841J	50S ribosomal protein L30	40.71

Table 6 Genes belonging to COG J with average degree bigger than 40 (see Figures 7 and 8). All these genes are conserved, common to all species (ERI=1), and drive the transition shown in Figure 6