Software

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Prolinks: a database of protein functional linkages derived from coevolution

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Abstract

The advent of whole-genome sequencing has led to methods that infer protein function and linkages. We have combined four such algorithms (phylogenetic profile, Rosetta Stone, gene neighbor and gene cluster) in a single database - Prolinks - that spans 83 organisms and includes 10 million high-confidence links. The Proteome Navigator tool allows users to browse predicted linkage networks interactively, providing accompanying annotation from public databases. The Prolinks database and the Proteome Navigator tool are available for use online at http://dip.doe-mbi.ucla.edu/pronav.

Rationale

Genome sequencing has allowed scientists to identify most of the genes encoded in each organism. The function of many, typically 50%, of translated proteins can be inferred from sequence comparison with previously characterized sequences. However, the assignment of function by homology gives only a partial understanding of a protein's role within a cell. A more complete understanding of protein function requires the identification of interacting partners: interacting subunits if the protein is a component of a molecular complex, and pathway members if the protein participates in a metabolic or signal transduction pathway [1]. Knowledge of these relationships, which we will call 'functional linkages', is a prerequisite for understanding physiology and pathology.

An enhanced understanding of the physical and functional relationships between proteins has recently become attainable through the use of non-homology-based methods [2,3]. These methods infer functional linkage between proteins by identifying pairs of nonhomologous proteins that coevolve. Evolutionary pressure dictates that pairs of proteins that function in concert are often both present or both absent within genomes (phylogenetic profiles method), tend to be coded nearby in multiple genomes (gene neighbors method), might be fused into a single protein in some organisms (Rosetta Stone method) or are components of an operon (gene cluster method). In contrast, proteins not related by function need not appear together or exhibit spatial proximity in the genome. The complete sequencing of over 100 genomes provides a rich medium from which to infer protein linkages and function by analyzing pairwise properties using these methods. Protein functional links may also be inferred from automated text mining. Here we use a simple algorithm (Text-Links) to identify proteins that are often found together in scientific abstracts [4].

In this paper we describe a new publicly available database - Prolinks - and the associated Proteome Navigator tool that combine pairwise associations generated from each of the inference methods mentioned above. This tool allows the user to explore interactively the protein links generated for 83 microbial organisms. Sequence, sequence homology, and public annotation, including the Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG) and National Center for Biotechnology Information (NCBI) descriptions, are available for each protein. The network of predicted associations is tunable, based on an adjustable confidence limit. The network has 'clickable' nodes that permit rapid navigation. Although this is not the first database that analyzes protein coevolution, it is in many respects distinct from existing tools [5,6]. In the Discussion section we analyze these differences. We also show how the Proteome Navigator may be used to recover links between functionally related proteins and between proteins contained within protein complexes. In short, this database extends the value of existing tools for genome annotation.

Genomic inference methods

The four genomic methods used by the Proteome Navigator are the phylogenetic profile, gene neighbor, Rosetta Stone, and gene cluster methods. An additional method, named TextLinks, does not use genomic context to infer functional linkages, but instead provides an automated analysis of PubMed scientific abstracts to infer protein relationships. Although each approach has been previously reported, here we provide the details of its implementation in the Prolinks database.

Phylogenetic profile method

The phylogenetic profile method uses the co-occurrence or absence of pairs of nonhomologous genes across genomes to infer functional relatedness [7,8]. The underlying assumption of this method is that pairs of nonhomologous proteins that are often present together in genomes, or absent together, are likely to have coevolved. That is, the organism is under evolutionary pressure to encode both or neither of the proteins within its genome and encoding just one of the proteins lowers its fitness. As in all of the above methods, we assume, and later confirm, that coevolved genes are likely to be members of the same pathway or complex.

Because sequenced genomes allow us to catalog most of the proteins encoded in each organism, we can determine the pattern of presence and absence of a protein by searching for its homologs across organisms. We define a homolog of a query protein to be present in a secondary genome if the alignment, using BLAST [9], of the query protein with any of the proteins encoded by the secondary genome generates an E-value less than 10^{-10}. The result of this calculation across N genomes yields an N-dimensional vector of ones and zeroes for the query protein that we call a phylogenetic profile. At each position in the profile the presence of a homolog in the corresponding genome is indicated with a one and an absence with a zero. A schematic representation of the construction of phylogenetic profiles is shown in Figure 1.

Using this approach we can readily compute the phylogenetic profiles for each protein coded within a genome of interest. We next need to determine the probability that two proteins have coevolved; this is based on the similarity of their profiles. If we assume that the two proteins A and B do not coevolve, we can compute the probability of observing a specific overlap between their two profiles by chance by using the hypergeometric distribution:

$$P\left(k' \mid n,m,N\right)=\frac{\begin{pmatrix} n \\ k \end{pmatrix}\begin{pmatrix} N-n \\ m-k \end{pmatrix}}{\begin{pmatrix} N \\ m \end{pmatrix}}$$

where N represents the total number of genomes analyzed, n the number of homologs for protein A, m the number of homologs for protein B and k' the number of genomes that contain homologs of both A and B [10]. Because P represents the probability that the proteins do not coevolve, 1 - $P(k > k')$ is then the probability that they do coevolve. We compute this probability for all pairs of proteins within a genome.

Gene cluster method

Within bacteria, proteins of closely related function are often transcribed from a single functional unit known as an operon. Operons contain two or more closely spaced genes located on the same DNA strand. These genes are often in proximity to a transcriptional promoter that regulates operon expression. Various methods have been developed to identify operon structure within microbial genomes [11-13], relying on intergenic distance as a predictor of operon structure.

Our approach to the identification of operons begins with the assumption that gene start positions can be modeled by a Poisson distribution, with each position having the same probability of being a start site. In other words, if we consider only the intergenic regions of a genome plus all the start sites, the probability that a gene starts at any position is given by $P(start) = me^{-m}$ where m is the total number of genes divided by the number of intergenic nucleotides. It follows that the probability that a gene does not start at a position is

$P(position\_without\_start) = e^{-m}$ and the probability of N - *1* sequential nucleotides without a start site followed by a start site is $P(N\_positions\_without\_starts) = me^{-Nm}$. From this we estimate the probability that two genes are separated by a distance less than N:

$$P\left(separation < N\right)=\int_{0}^{x} me^{-mN} = 1-e^{-mx}$$

We assume that the probability that two genes that are adjacent and coded on the same strand are part of an operon is *1* - *P*, as the more likely we are to find a greater intergenic

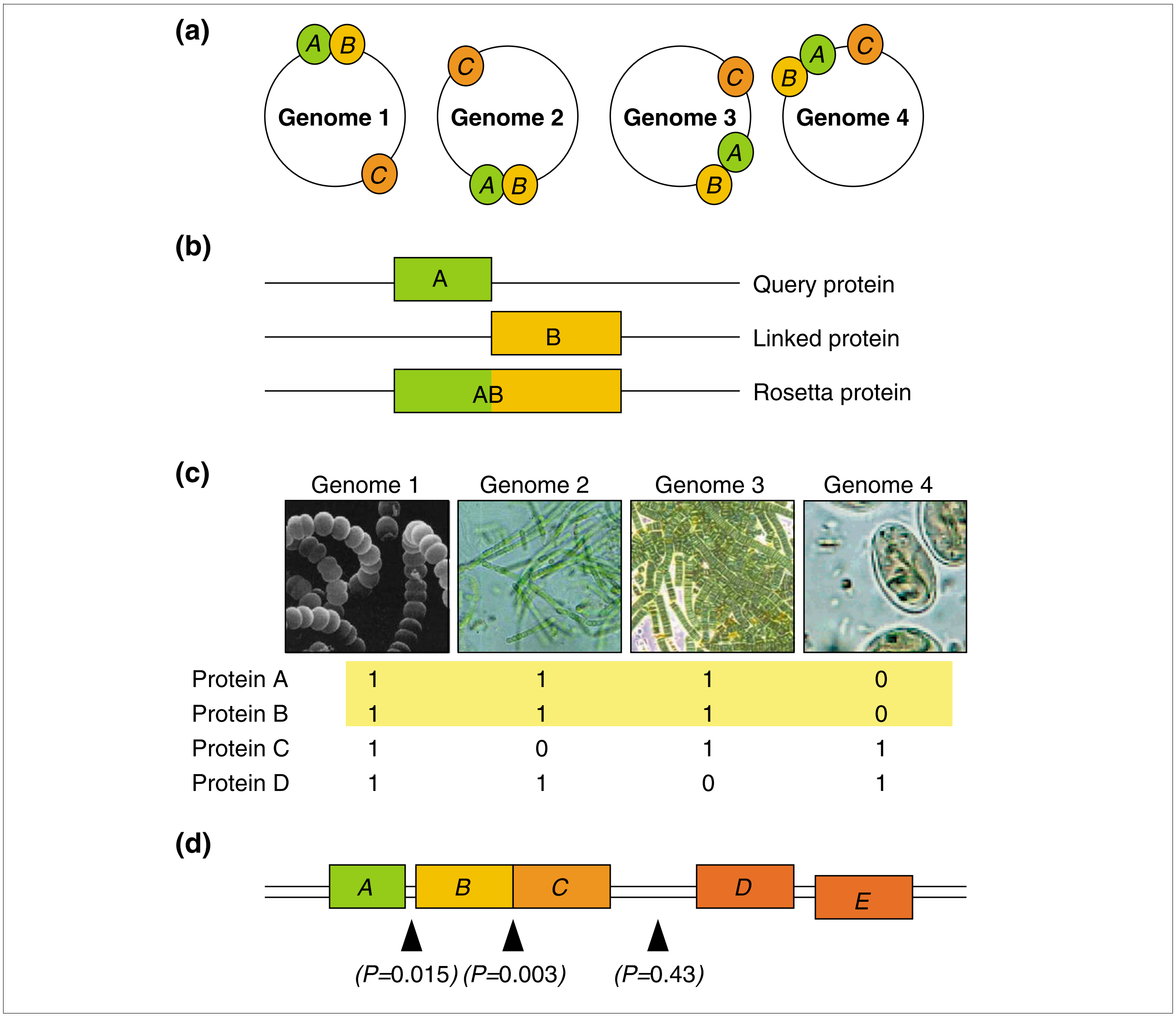


Figure 1
The general mechanism of inference for each of the four methods used by the Proteome Navigator. **(a)** The gene neighbor (GN) method identifies protein pairs encoded in close proximity across multiple genomes. We see in this example that genes *A* and *B* are gene neighbors while *A* and *C* are not. **(b)** The Rosetta Stone (RS) method searches for gene fusion events. We see that the A and B proteins are expressed as separate proteins in one organism. However, in a second organism a sequence exists that represents the fusion of the two proteins. The fusion protein is termed the Rosetta Stone protein as it allows us to infer that the A and B proteins are functionally linked. **(c)** The construction of phylogenetic profiles (PP) begins with four sequenced genomes, from which the protein sequences have been predicted. The protein sequence, A, within *E. coli* is compared to that of the proteins coded by the other genomes and homologs are identified. If the genome contains a homolog of A, a 1 is placed in the corresponding phylogenetic profile position, a 0 otherwise. Genes with similar phylogenetic profiles are likely to participate in the same pathway. **(d)** The gene cluster (GC) or operon method identifies closely spaced genes, and assigns a probability *P* of observing a particular gap distance (or smaller), as judged by the collective set of inter-gene distances.

separation the less likely two genes are to be part of an operon. Although this is a very simple model of intergenic spacing, it captures the basic biology that the closer two co-directional genes are, the more likely they are to be members of the same operon. Unlike the other coevolution methods described here, the gene cluster method is able to identify potential functions for proteins exhibiting no homology to proteins in other genomes.

Gene neighbor method

Some of the operons contained within a particular organism may be conserved across other organisms. The conservation of an operon's structure provides additional evidence that the genes within the operon are functionally coupled and are perhaps components of a protein complex or pathway. Several methods have been reported that identify conserved operons [14-16]. However, unlike the previous approaches, we have developed a novel algorithm that generates a *P* value for the likelihood that two proteins are coded within a conserved operon. A schematic describing this method is shown in Figure 1, where genes A and B are found in close proximity on four genomes, while gene C is positioned randomly.

Our approach, the gene neighbor method, first computes the probability that two genes are separated by fewer than *d* genes:

$$P(\leq d) = \frac{2d}{N-1}$$

where *N* is the total number of genes in the genome. Note that we must use the smaller of two values of *d* for two genes that are coded on a circular plasmid or circular chromosome. If the two genes have homologs in other organisms we compute the product of the above probability across these organisms:

$$X = \prod_{i=1}^{m} P_i(\leq d_i) = \prod_{i=1}^{m} \frac{2d_i}{N_i - 1}$$

where *m* is the number of organisms that contain homologs of the two genes of interest.

To compute the likelihood that two genes are components of a conserved operon we need to compute the probability of obtaining a value of *X* that is smaller than the observed value. It can be shown that this probability is given by:

$$P_m(\leq X) = 1 - P_m(> X) \approx X \sum_{k=0}^{m-1} \frac{(-\ln X)^k}{k!}$$

Rosetta Stone method

Occasionally, two proteins expressed separately in one organism can be found as a single chain in the same or a second genome. Analysis of gene fusion/division events to infer functional relatedness, commonly known as the Rosetta Stone method, is illustrated in Figure 1, and has been described in detail elsewhere [17,18]. Proteins that carry out consecutive metabolic steps or are components of molecular complexes are often expressed as a single polypeptide chain to maximize kinetic or expression efficiency.

To detect gene-fusion events we first align all protein-coding sequences from a genome against the nonredundant database using BLAST. We identify cases where two nonhomologous proteins both align over at least 70% of their sequence to different portions of a third protein. We refer to the third protein as the Rosetta Stone protein. When this situation arises we hypothesize that during the course of evolution the ancestors of the two proteins fused to form the ancestor of the Rosetta Stone protein.

A confounding aspect of this analysis is that many of the alignments between the starting proteins and the Rosetta Stone protein occur in regions of highly conserved domain sequences, such as kinase or zinc finger domains. Proteins that contain these common domains are often found linked to each other by the Rosetta Stone method, even though they may not have fused.

To screen out these confounding fusion events we compute the probability that two proteins are found linked by the Rosetta Stone method by chance alone:

$$P(k' \mid n, m, N) = \frac{\binom{n}{k}\binom{N-n}{m-k}}{\binom{N}{m}}$$

where *k'* is the number of Rosetta Stone sequences, *n* the number of homologs of protein A and *m* the number of homologs of protein B and *N* the total number of sequences in the nr database [19]. In other words, if a protein has many homologs in the database, possibly because it contains a common domain, it is likely to be linked to a second protein, even though the Rosetta Stone protein did not evolve by a fusion of this protein with another. Therefore, the probability that two proteins have fused is given by 1 - *P*(*k* >*k'*).

TextLinks

Just as the systematic presence or absence of coevolved genes across genomes can be used to infer functional linkages, so to can the co-occurrence of gene names and symbols within the scientific literature be used to establish known gene interactions. Again, the underlying assumption is that genes, related by function, will often appear within the same scientific article or abstract. For this analysis, we have used the PubMed database [20], containing 14 million abstracts and citations, as a basis set. Within abstracts, we identify the presence or absence of individual genes using a controlled vocabulary of gene names and symbols available for each genome at NCBI [21].

As with the phylogenetic profile method, abstracts and individual gene names were used to develop a binary vector describing each protein's distribution within the scientific literature. The result is an *N*-dimensional vector (where *N* is the total number of abstracts) of ones (a protein name is found within a given abstract or citation) and zeroes (the protein name is absent) for the query protein. Using this approach, we compute the literature profile for each protein coded within a genome of interest. Finally, we compute the probability that two proteins are related, based on the similarity of their literature profiles, using the same hypergeometric distribution function used for the phylogenetic profile and Rosetta Stone methods:

$$P\left(k' \mid n,m,N\right)=\frac{\binom{n}{k}\binom{N-n}{m-k}}{\binom{N}{m}}$$

where N represents the total number of abstracts analyzed, n the number of instances for the protein A name or symbol, m the number of instances for the protein B name or symbol, and k' the number of abstracts that contain both A and B protein names or symbols. The probability that two proteins are literature related, given as 1 - $P(k > k')$, is computed for all pairs of annotated proteins within a genome. TextLinks represents an attempt to mine the current state of scientific understanding of protein function and interactions. Currently TextLinks are available within Prolinks and the Proteome Navigator only for *E. coli*.

The Prolinks database

Each of the methods outlined above is statistical in nature, allowing us to compute a probability associated with each predicted interaction. However, the probability metrics from different methods differ in scale, making direct comparison of inference between methods problematic. To overcome this limitation we have developed a universal confidence metric.

The confidence metric for each prediction is derived from COG pathway recovery [22]. For each method, inferences are ordered by their intrinsic statistical metric (P-value) and the cumulative accuracy with which COG pathway annotation is recovered, starting from the most significant prediction, is recorded for each pairwise prediction. Recovery means that both proteins belong to the same pathway. Predicted pairs with the same COG pathway annotation are treated as true positive, while pairs assigned to different COG pathways are considered false positive.

The current version of the Prolinks database contains linkages for 83 genomes. We list all the organisms in Table 1: there are ten from the Archaea, five from the Eukaryota and the rest are from the Bacteria. In total we have computed 18,077,293 links between proteins coded within these genomes. As the number of fully sequenced genomes is constantly increasing, we expect that future versions of this database will contain significantly more data. The Prolinks database may be accessed though the Proteome Navigator tool [23] or by accompanying flatfiles.

Figure 2 shows how well each of the four coevolution methods performs in recovering protein pairs that are assigned to the same COG pathway. Based on this metric, the gene neighbor method provides the most accurate and extensive coverage of the four methods, whereas the gene cluster method is the least accurate.

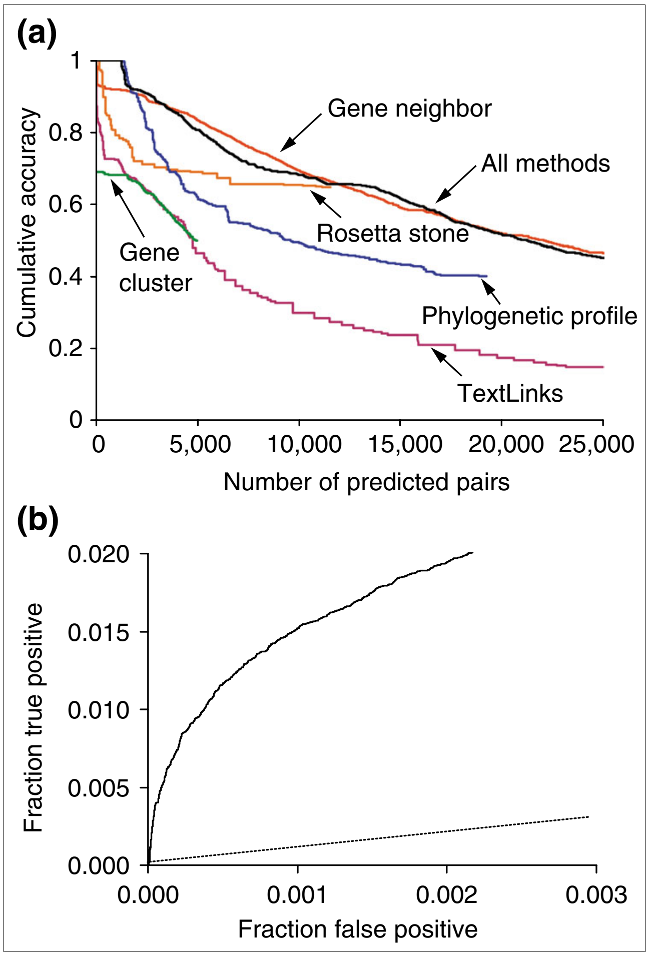


Figure 2
We assess COG category recovery for the four individual methods, the combination of the four methods, and TextLinks. **(a)** We assign a confidence measure to the likelihood that a pair of proteins is acting within the same COG pathway, reflecting the number of COG-annotated pairs that lie within the same pathway relative to the total number of annotated pairs. The COG confidence metric is used in the network-graphing function of the Proteome Navigator to select inferred protein linkages with uniform confidence. *E. coli* protein pairs displayed in this figure have a COG pathway confidence recovery (cumulative accuracy) of greater than 0.4, with the exception of the TextLinks pairs. **(b)** The receiver operator characteristic (ROC) curve shows the performance of the rank-ordered list of all *E. coli* interactions predicted from genomic inference (solid line) compared with the random selection of protein pairs (dashed line).

Because each method is now measured according to the same confidence metric, we combine all the methods by considering any pair of genes to be linked with a confidence given by the maximal confidence of any method. The receiver operator characteristic curve (ROC; Figure 2b) shows that the rank-ordered list of combined protein interactions recovers functionally related protein links with a 15-fold greater accuracy than would be expected from a random selection of protein pairs. From this analysis we conclude that pairs of genes that function within the same pathway are likely to be coupled

Table 1

Genomes contained in Prolinks

Taxonomy ID	Name	Lineage
24	*Shewanella putrefaciens*	Bacteria
139	*Borrelia burgdorferi*	Bacteria
158	*Treponema denticola*	Bacteria
160	*Treponema pallidum*	Bacteria
197	*Campylobacter jejuni*	Bacteria
287	*Pseudomonas aeruginosa*	Bacteria
303	*Pseudomonas putida*	Bacteria
358	*Agrobacterium tumefaciens*	Bacteria
382	*Sinorhizobium meliloti*	Bacteria
485	*Neisseria gonorrhoeae*	Bacteria
520	*Bordetella pertussis*	Bacteria
601	*Salmonella typhi*	Bacteria
632	*Yersinia pestis*	Bacteria
666	*Vibrio cholerae*	Bacteria
714	*Actinobacillus actinomycetemcomitans*	Bacteria
747	*Pasteurella multocida*	Bacteria
782	*Rickettsia prowazekii*	Bacteria
837	*Porphyromonas gingivalis*	Bacteria
881	*Desulfovibrio vulgaris*	Bacteria
920	*Acidithiobacillus ferrooxidans*	Bacteria
956	*Wolbachia* sp.	Bacteria
1097	*Chlorobium tepidum*	Bacteria
1148	*Synechocystis* sp. PCC 6803	Bacteria
1299	*Deinococcus radiodurans*	Bacteria
1309	*Streptococcus mutans*	Bacteria
1313	*Streptococcus pneumoniae*	Bacteria
1314	*Streptococcus pyogenes*	Bacteria
1351	*Enterococcus faecalis*	Bacteria
1352	*Enterococcus faecium*	Bacteria
1360	*Lactococcus lactis* subsp. *lactis*	Bacteria
1392	*Bacillus anthracis*	Bacteria
1423	*Bacillus subtilis*	Bacteria
1488	*Clostridium acetobutylicum*	Bacteria
1496	*Clostridium difficile*	Bacteria
1717	*Corynebacterium diphtheriae*	Bacteria
1764	*Mycobacterium avium*	Bacteria
1769	*Mycobacterium leprae*	Bacteria
1772	*Mycobacterium smegmatis*	Bacteria
1773	*Mycobacterium tuberculosis*	Bacteria
2097	*Mycoplasma genitalium*	Bacteria
2104	*Mycoplasma pneumoniae*	Bacteria
2107	*Mycoplasma pulmonis*	Bacteria
2130	*Ureaplasma urealyticum*	Bacteria
2190	*Methanocaldococcus jannaschii*	Archaea
2234	*Archaeoglobus fulgidus*	Archaea
2287	*Sulfolobus solfataricus*	Archaea
2303	*Thermoplasma acidophilum*	Archaea
2336	*Thermotoga maritima*	Bacteria

Table 1 *(Continued)*

Genomes contained in Prolinks

2371	*Xylella fastidiosa*	Bacteria
3702	*Arabidopsis thaliana*	Eukaryota
4932	*Saccharomyces cerevisiae*	Eukaryota
5476	*Candida albicans*	Eukaryota
6239	*Caenorhabditis elegans*	Eukaryota
7227	*Drosophila melanogaster*	Eukaryota
29292	*Pyrococcus abyssi*	Archaea
35554	*Geobacter sulfurreducens*	Bacteria
50339	*Thermoplasma volcanium*	Archaea
53953	*Pyrococcus horikoshii*	Archaea
56636	*Aeropyrum pernix*	Archaea
61435	*Dehalococcoides ethenogenes*	Bacteria
63363	*Aquifex aeolicus*	Bacteria
64091	*Halobacterium* sp. NRC-1	Archaea
69394	*Caulobacter vibrioides*	Bacteria
71421	*Haemophilus influenzae* Rd KW20	Bacteria
83331	*Mycobacterium tuberculosis* CDC1551	Bacteria
83333	*Escherichia coli* K12	Bacteria
83334	*Escherichia coli* O157:H7	Bacteria
83554	*Chlamydophila psittaci*	Bacteria
83560	*Chlamydia muridarum*	Bacteria
85962	*Helicobacter pylori* 26695	Bacteria
85963	*Helicobacter pylori* J99	Bacteria
86665	*Bacillus halodurans*	Bacteria
107806	*Buchnera aphidicola* str. APS	Bacteria
115711	*Chlamydophila pneumoniae* AR39	Bacteria
115713	*Chlamydophila pneumoniae* CWL029	Bacteria
122586	*Neisseria meningitidis* MC58	Bacteria
122587	*Neisseria meningitidis* Z2491	Bacteria
129958	*Carboxydothermus hydrogenoformans*	Bacteria
138677	*Chlamydophila pneumoniae* J138	Bacteria
145262	*Methanothermobacter thermautotrophicus*	Archaea
155864	*Escherichia coli* O157:H7 EDL933	Bacteria
158878	*Staphylococcus aureus* subsp. *aureus* Mu50	Bacteria
158879	*Staphylococcus aureus* subsp. *aureus* N315	Bacteria

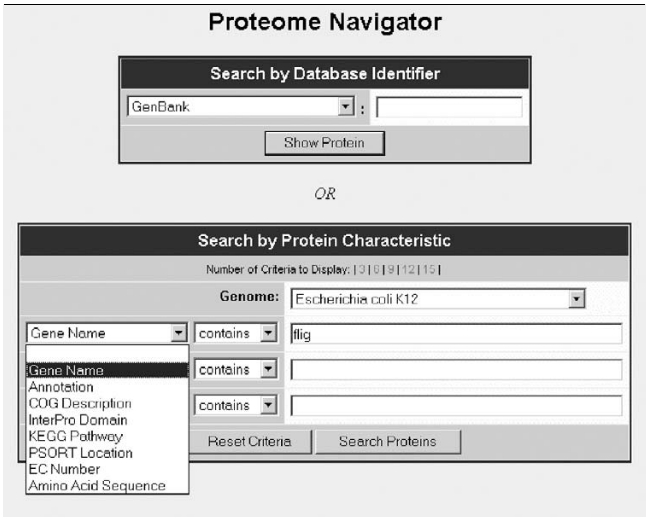
during the course of their evolution. Therefore the methods we have developed to infer coevolution between proteins are useful for detecting protein pairs that act within the same cellular pathways.

Proteome Navigator

We applied the four genomic inference methods to 83 fully sequenced microbial genomes and the TextLinks approach to *Escherichia coli*. The resulting calculation generates several hundred thousand predicted protein associations for each organism. In order to facilitate access to these data, we have developed an online browser, the Proteome Navigator [23].

The opening page of the Proteome Navigator prompts the user to identify a protein using a protein name, sequence identifier or functional category (Figure 3). Note that if a protein is selected on the basis of an identifier, it may not be coded within a fully sequenced genome contained in the database; in which case no Prolinks will be generated for the protein. To identify a related gene or gene name that is coded within a fully sequenced genome, one may use BLAST against the fully sequenced genome at NCBI.

Selecting an individual protein takes the user to a general protein information page, providing the protein's primary



GenBank :

Genome: Escherichia coli K12

Gene Name contains flig

Figure 3
The opening page of the Proteome Navigator prompts the user to select a protein by database identifier or protein name or ID, as well as selecting the genome of interest. Pull-down tabs facilitate the selection of protein features and microbial genomes. Here we select the *E. coli* gene '*fliG*'. Clicking the 'Search Proteins' button takes the user to a page displaying all of the proteins that satisfy the search criteria (see Figure 4).

sequence, known function(s), name and alias. Tabs at the top of each page allow the user to examine known homologs of the protein, the profile or distribution of homologous proteins among the sequenced genomes, protein characteristics and annotation, and the graph of the network of predicted interactions for the protein.

The graph function of the Proteome Navigator (Figure 4) allows the user to navigate the network of predicted interactions interactively. The layout of the graph is determined using a spring minimization algorithm. Each protein is connected by a 'spring' whose spring constant is proportional to the number of links separating the nodes on the graph. Because the minimization algorithm is seeded with a random number, each time the graph is rerun it will generate a different layout.

The graph tab also permits the user to vary the scope and attributes of the resulting network. For instance, the 'graph order' function can be used to extend the network to include all proteins that are linked within *n* interactions of the input seed protein. Higher graph orders generate networks of increasing size and complexity. A setting of graph order of 2 prompts the Proteome Navigator to first identify protein links satisfying a minimum confidence threshold to an original search protein. This original group of identified proteins is then used to perform a secondary search using the same criteria. The original protein is displayed in the resulting network as a double-lined box located towards the center of the graph. An example of such a second-order search and the resulting network is shown in Figure 4, highlighting the *E. coli* flagellar complex.

Additional graphing capabilities are also available, including coloring of the protein nodes based on known KEGG or COG pathway annotation and 'clickable' protein nodes. Clicking on a given protein node within the displayed network prompts the Proteome Navigator to perform a new search using the chosen node as the beginning search protein and the same search parameters as before. This operation allows the user to navigate easily through the entire microbial network without manually selecting new protein-search criteria.

Another important feature of the Proteome Navigator allows one to obtain detailed information on each link. In the Prolinks tab all of the links associated with the starting protein are listed. Associated with each link is a 'detail' hyperlink that generates a separate browser page that describes the underlying source for each link. For instance, in the case of phylogenetic profile links, the page reports the organisms that contain the two proteins of interest, and the probability of finding the observed number of matches between the two profiles.

Example results

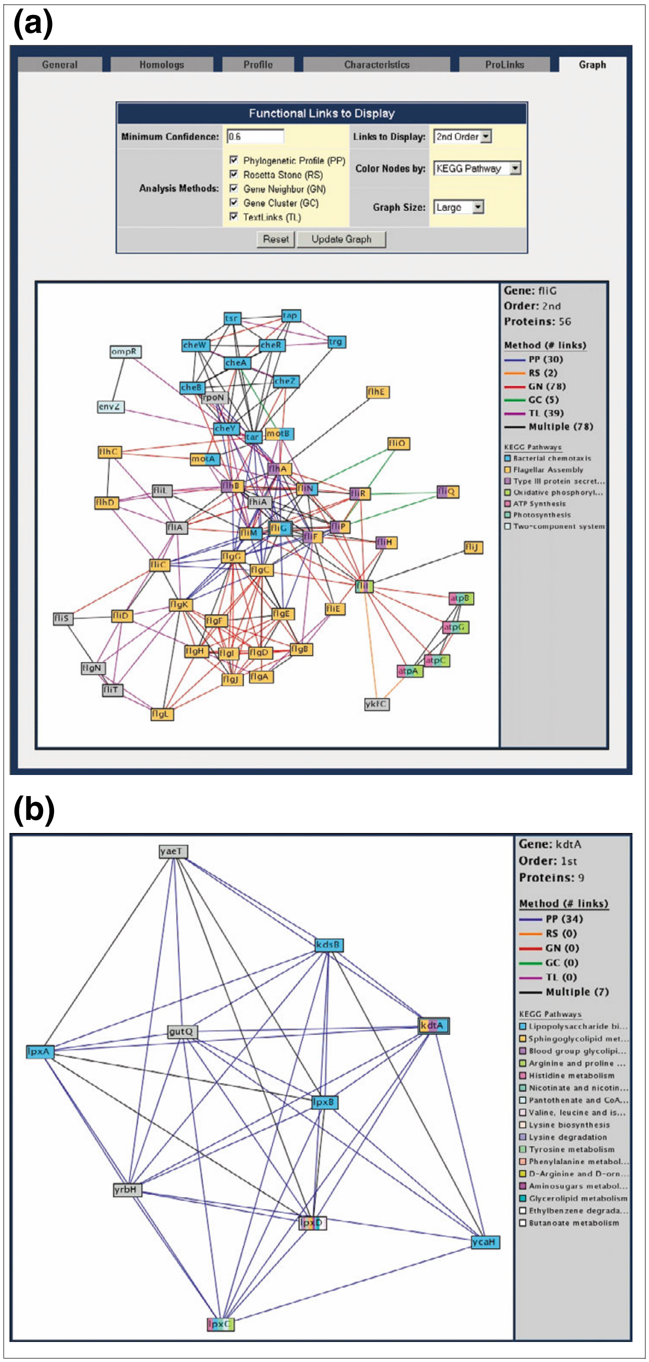
Chemotaxis

To illustrate the utility of the Proteome Navigator, we show a network search starting with a known member of the *E. coli* flagellar assembly, FliG. Specifying a confidence metric of 0.6 and graph degree setting of 2, we obtain the network shown in Figure 4, colored by KEGG pathway categories.

In addition to identifying most components of flagellar biosynthesis, control and structure (FliS, Flit, FliA, FliL, FliA, and so on; orange), this procedure also associates subnetworks of related function. These include the flagellar ATP synthase complex (AtpA, AtpC, AtpB, AtpG, FliI; red, green, blue), chemotaxis (CheR, CheB, CheY, CheZ, Tar, Tap; blue), cell motility (MotA, MotB, CheA, CheW; blue), and osmolarity sensors (OmpR, EnvZ; aqua). Each functional category sublocalizes within the network, providing an intuitive summary of the *E. coli* chemotaxis multiprotein complexes and their interrelationships.

Previously uncharacterized proteins such as YkfC, shown in gray in Figure 4, also appear within the network. We see that YkfC has multiple links to the bacterial chemotaxis machinery and would therefore predict it to have a function related to chemotaxis. We note that YkfC has no sequence similarity to the other chemotactic proteins. Hence this putative functional relationship has been discovered by non-homology methods.

We also note that the network also contains some false-positive links. For instance, although OmpR and CheY are linked



Functional Links to Display

Minimum Confidence: 0.6

Links to Display: 2nd Order

Analysis Methods:
☑ Phylogenetic Profile (PP)
☑ Rosetta Stone (RS)
☑ Gene Neighbor (GN)
☑ Gene Cluster (GC)
☑ TextLinks (TL)

Color Nodes by: KEGG Pathway

Graph Size: Large

Gene: fliG
Order: 2nd
Proteins: 56

Gene: kdtA
Order: 1st
Proteins: 9

Figure 4
The 'Graphing' function of the Proteome Navigator displays the network of interactions satisfying the input search criterion. **(a)** Nodes are colored by functional categories explained in the right-hand border. Edges connecting proteins are colored by the method predicting the interaction, also described in the figure border. Associations predicted by multiple methods are shown in black. The double box around fliG indicates that this was the input protein used to generate this network. Clicking on a node brings the user to a protein-annotation page, and the search can be continued using the new protein to generate a new network search. **(b)** An example of functional discovery using Prolinks. Using *kdtA* as the initial seed, we speculate that GutQ, an uncharacterized *E. coli* protein, may be associated with lipopolysaccharide and cell-wall synthesis. Confirmation of these predictions awaits further scientific inquiry.

by TextLinks, they are not in fact associated. The linkage is derived from the fact that the two proteins often appear together in abstracts, despite the fact that they do not physically associate.

This example illustrates one possible use of predicted networks, which is to the assign a function to uncharacterized genes [24-26]. In the case of *E. coli*, only two thirds of the genes have been functionally annotated, according to the NCBI documentation. This leaves 30% of genes with no functional annotation using any of the standard homology-based bioinformatics techniques. Using Prolinks, we can assign putative functions to most of these 1,500 open reading frames (ORFs).

Lipopolysaccharide biosynthesis example

Another example that demonstrates the pathway reconstruction and function assignment capabilities of Prolinks involves the lipolysaccharide biosynthesis pathway. This pathway contains proteins that are involved in the formation from simpler components of lipopolysaccharides, any of a group of related, structurally complex components of the outer membrane of Gram-negative bacteria.

In Figure 4b we show a network seeded with the lipolysaccharide pathway gene *kdtA* (3-deoxy-D-manno-octulosonic-acid transferase). This network involves six genes known to be involved in the pathway. Along with the known genes we also find other uncharacterized ORFs (*gutQ*, *yrbH* and *yaeT*) that are also tightly linked to the cluster. We postulate from this analysis that all three of these genes are likely to be involved with the lipolysaccharide biosynthesis pathway.

Protein complexes

While the ability of coevolution methods to identify functionally related proteins has been well established, it has been less clear how well they recognize direct protein interactions. We show here that the methods are very effective in identifying interactions between subunits of protein complexes. We used the EcoCyc library of *E. coli* multiprotein complexes [27] to assess the ability of the Proteome Navigator to identify direct protein physical interactions.

Figure 5 illustrates the performance of each of the four methods in identifying components of multiprotein complexes. In contrast to COG pathway benchmarking, gene cluster performs best among the methods, identifying 6,000 protein interactions with greater than 83% accuracy, as judged by the EcoCyc benchmarking. The phylogenetic profile method identifies members of known *E. coli* complexes with an accuracy of 30% (greater than the 1% percent accuracy random selection would provide), but the accuracy appears to be independent of the statistical confidence (*P*-value) of the prediction. On the basis of the totality of these benchmarking results, the Prolinks database performs well in identifying subunits of protein complexes.

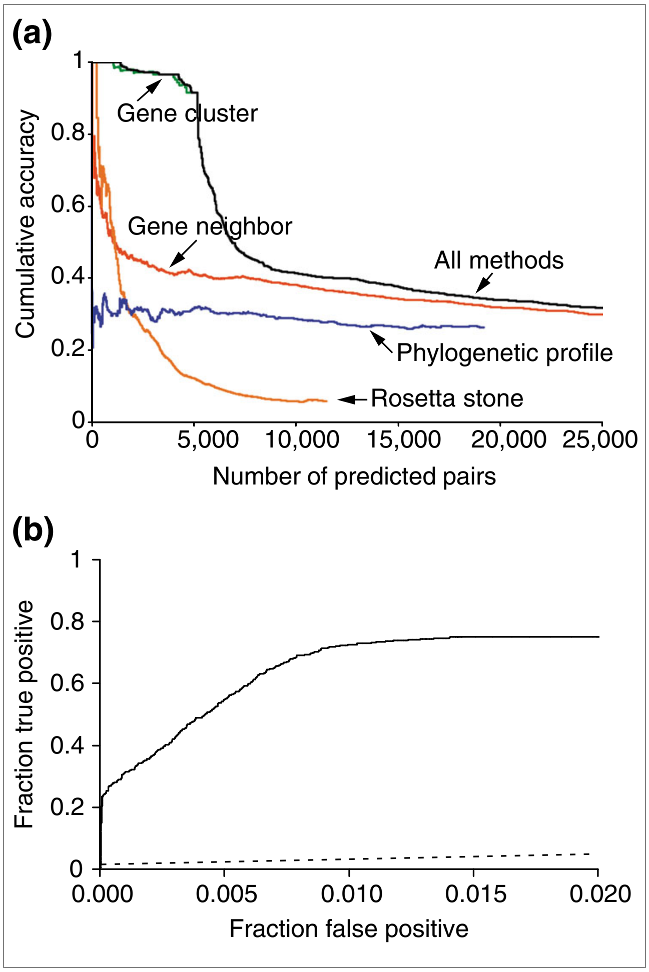


Figure 5
Assessment of the four methods by recovery of links between members of known *E. coli* protein complexes. **(a)** We test to see how often predicted interacting protein pairs are subunits of the same protein complex. *E. coli* protein complexes were obtained from the EcoCyc database. **(b)** Again, the ROC curve shows the performance of the rank-ordered list of all *E. coli* predicted interactions (solid line) compared with the random selection of protein pairs (dashed line), in their ability to recover constituents of known protein complexes.

Existing coevolution databases

Two databases previously described as compiling information on coevolving proteins are Predictome [5] and String [6]. Although these databases use some of the same methods described here, they differ from Prolinks in some important respects.

Predictome, for instance, uses the gene fusion and phylogenetic methods to predict interactions between proteins. However, unlike Prolinks, there appear to be no statistical measures to gauge the accuracy of each prediction. This is potentially a significant limitation because, as we show in Figure 2, the accuracy with which these methods recover known pathway associations changes dramatically as a function of the *P*-value.

Unlike the Predictome database, the String database does produce a score to estimate the accuracy of each pairwise association. However, unlike the Prolinks database, which is based on single proteins in a specific genome, the String database is constructed around COGs [22]. COGs are groups of orthologous proteins across organisms that have been determined using sequence-alignment techniques. The use of COGs rather than individual genes has both benefits and limitations. One of the limitations, as we will see in the example below, is that the analysis generates a COG network that includes COGs that may not be present in the organism you are interested in. Another difference between the two databases is that Prolinks attempts to reconstruct the operon structure of each organism, while String relies only on the other three methods.

Comparative benchmarking of databases

To compare Prolinks to the String and Predictome databases we have downloaded all the functional links for *E. coli* in each database. We obtained 407,520 links from String and 22,004 from Predictome in comparison with 515,892 links from coevolution methods from Prolinks (that is, not including TextLinks). For the links from String and Predictome, we could not rank order the linkages as no quality measure is provided. Therefore, in all cases we compute only averages for the entire list.

To assess the quality of the lists, we computed the fraction of links between proteins assigned to COG pathways that are between proteins in the same pathway. In the case of String we found that 17% of the annotated links were between proteins in the same pathway. When we took the top 407,000 links between *E. coli* proteins in Prolinks, we found that 20% of the links between proteins assigned to a COG pathway were between proteins in the same pathway.

Similarly, we also calculated the fraction of annotated links that are between proteins in the same COG pathway for the Predictome list of 22,004 links. In this case we found that 60% of the links were between intrapathway pairs. We compared this fraction to that obtained from the top 22,000 Prolinks linkages that gave 68%.

The conclusion from both these analyses is that by these measures Prolinks predicts more physical and functional linkages at higher accuracy than those presently contained in the String and Predictome databases. Because COG pathways were not used to generate the linkages, this is a rigorous test of the capability with which linkages associate members of the same pathway. We also note that Prolinks contains more than ten times as many linkages as the Predictome database

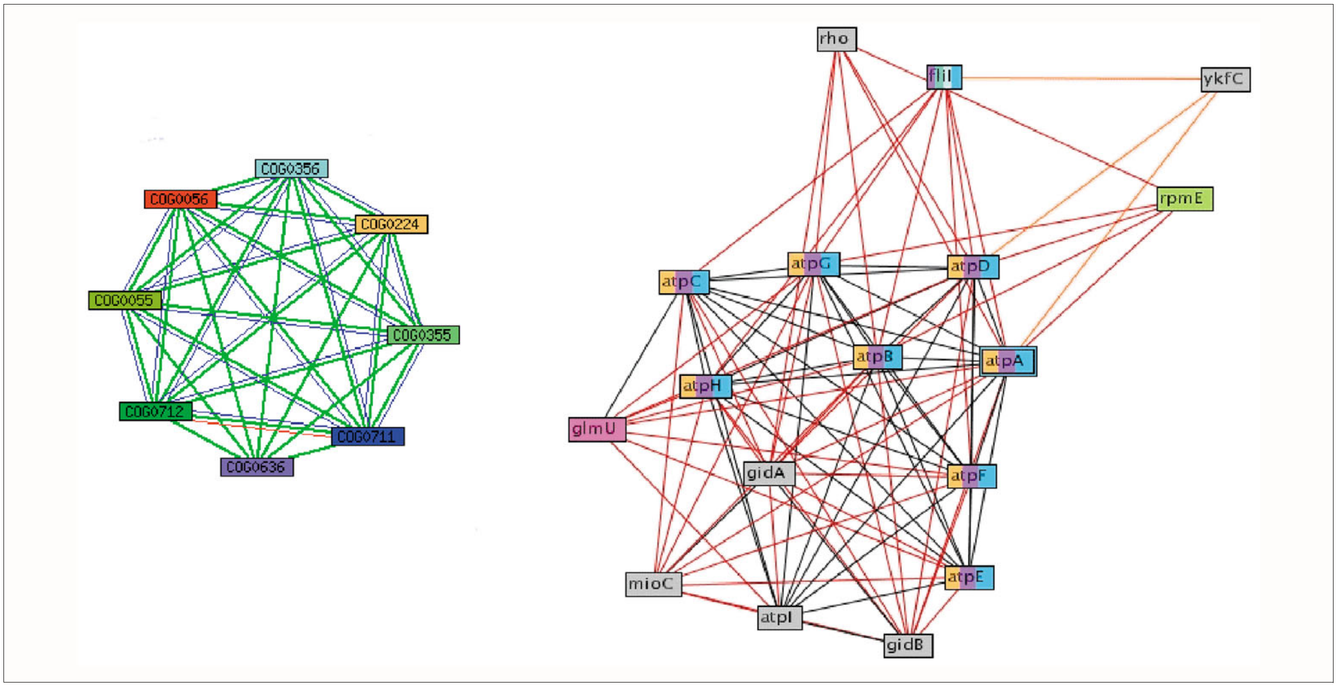


Figure 6
A comparison of graphs generated by querying the String database and Proteome Navigator to identify proteins in the ATP synthase complex. COG0056, shown in red in the String network (left), contains the *E. coli* protein AtpA, used to search each database and shown highlighted as a double-lined box in the Proteome Navigator graph (right). The Proteome Navigator network and Prolinks database identify twice the number of functionally linked proteins at the given confidence level.

and 25% more than the String database. Also, every link from Prolinks comes with a quality measure assigned.

We also used the EcoCyc database of known *E. coli* complexes to test the frequency with which the three linkage lists associate subunits of known complexes. In the case of String we find that 4% of the linkages between proteins that are subunits of complexes are between subunits of the same complex. In contrast, 9% of the top 407,000 Prolinks linkages between subunits of complexes are intra-complex pairs. We also find that 30% of the Predictome linkages between subunits of complexes are intra-complex pairs whereas 32% of the top 22,000 Prolinks linkages are between subunits of the same complex.

In conclusion, on the basis of a comparison with linkages from *E. coli*, we find that: Prolinks offers a greater number of functional linkages than other databases; each link from Prolinks is assigned a confidence measure; and that our benchmarking reported here of Prolinks against COG pathways or complexes compares favorably to the linkages contained in String and Predictome.

ATP synthase networks from Prolinks and String

Finally, we provide a side-by-side comparison of the String and Prolinks databases, and their ability to identify known and novel protein interactions within the *E. coli* genome. We begin by using identical input parameters, which include a 1-degree depth search, a 0.4 confidence setting, starting from the protein AtpA. Each graph in Figure 6 identifies seven additional members of the ATP synthase complex, including AtpB, AtpC, AtpD, AtpE, AtpF, AtpG and AtpH. The Proteome Navigator also identifies nine protein interactions not identified by the String database. For instance, FliI, a flagellar-specific component of the ATP synthase machinery, is not found by in the String search, but is linked to the search protein AtpA, as well as five other components of the ATP synthase complex, by the Prolinks database. The Proteome Navigator also predicts functional links to proteins known to govern *E. coli* energy metabolism, GidB and GidA, and other proteins of known chemotaxis- related function.

Perhaps more important, the Proteome Navigator identifies functional links to proteins of unknown function. In this instance, YkfC, an uncharacterized reverse transcriptase in *E. coli*, is linked to AtpA, FliI and AtpD, each suggesting that this protein may have a crucial role in the regulation of chemotaxis and motility. Small changes in the input parameters reveal four more uncharacterized proteins, as well as additional related chemotaxis and osmolarity sensor subnetworks, that are not found by an equivalent search using String.

A final and substantial difference between the respective databases is their ability to generate genome-specific graphs. Because the String database uses a COG-based approach to phylogenetic analysis and visual output, the information presented often contains linkages to COGs that are not present in the starting organism. For instance, a starting search using the *E. coli* gene *fliG* and a confidence limit of 0.4 identifies the protein as belonging to COG1536. The linkage analysis by String links COG1536 to COG1315, a predicted polymerase family not present within *E. coli* yet included within the resulting network. Graphs and linkages produced by the Proteome Navigator are always specific to the input organism and protein, producing graphs that contain nodes colored and clustered by known functional annotation, making their interpretation intuitive and ideal for discovery.

In conclusion, Prolinks complements existing databases and provides additional features and capabilities that are not found in Predictome and String. As such, we believe that Prolinks represents a useful addition to the suite of tools that are available to biologists to study protein functional linkages.

Discussion

Over the past few years significant progress has been made to measure protein interactions and protein complexes in cells using experimental approaches. Although the data have proved valuable to biologists, they are still limited in their coverage of organisms whose genomes have been fully sequenced. The majority of protein interactions have been measured within a single organism, *Saccharomyces cerevisiae* [28]. Although there is some value in extrapolating interactions from one organism to another using homology, several lines of evidence indicate that such an approach may be error-prone [29,30]. Furthermore, the underlying interaction data in a single organism has been shown to contain a large percentage of false positives [30].

To complement the directly measured data on protein interaction we have presented a comprehensive database of protein interactions inferred from 83 fully sequenced organisms by coevolutionary methods. We have shown that the computational methodology that we utilize to identify inferred interactions is able to link proteins that function within the same biochemical pathway as well as subunits of protein complexes.

The potential uses of these inferred functional linkages are several. By combining pairs of inferred linkages within a genome, one can build up networks of functional links. These give information on both protein complexes and metabolic pathways that can be compared with more directly measured information. The networks place proteins in their functional contexts in the cell, and can thus be used to gain an expanded view of the multiple functions of proteins within cells. This expanded view is readily accessible in the Prolinks database, and conveniently explored with the Proteome Navigator.

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