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REVIEW ARTICLE

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The evolution of function: a new method to assess the phylogenetic value of ribosomal sensitivity to antibiotics

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Summary Thirty-five archaeal, bacterial and eukaryotic translational systems have been proved against forty different protein synthesis inhibitors with diverse domain and functional specificities. The inhibition curves generated in every ribosome-antibiotic combination had previously shown interesting similarities among organisms belonging to the same phylogenetic group. This opened the possibility of using such functional information for developing evolutionary studies. A new mathematical method based on the main data components analysis has been developed to extract most of the information contained in the inhibition curves. The phenograms obtained closely resemble those generated by the small ribosomal subunit rRNA sequence comparison and such functional clustering is also congruent when a particular subset of organisms and/or antibiotics is used. These results prove the phylogenetic value of our functional analysis and suggest that the ribosome represents an interesting intersection between genotypic and phenotypic (functional) information stored in organisms.

Key words Phylogeny · Functional evolution · Ribosome · Protein synthesis inhibitors · Functiotype

Introduction

Before the discovery of the molecular basis of inheritance, morphological, physiological and behavioral diversity provided the only analyzable characters for systematics [31]. However, during the last forty years, the development of molecular biology has produced a radical change in evolutionary studies. Since a large amount of phylogenetic information is stored in the genomes of organisms, an increasing capacity of the techniques to analyze the genomes has been developed [25]. On the basis of the pioneering work of Zuckerkandl and Pauling [52], who provided the first indications of a molecular clock, the concept that semantophoretic molecules (DNA, RNA or proteins) can be used as molecular chronometers has been widely accepted. Those molecules measure not only evolutionary relationships but also the approximate time of divergence [47].

The advances in nucleic acids sequencing techniques, mainly applied to ribosomal RNA, have converted the comparison of homologous genes into one of the most powerful molecular approaches for inferring phylogenetic history [48]. Nevertheless, controversies have arisen among evolutionary biologists with regard to several problems related to the sequence analysis and its phylogenetic value. First, it is commonly accepted that there must be a close correlation between sequence divergence and time, but it is clear that functional constraints do not allow for evolutionary rates to be constant among all molecules [7], or even among the domains of a given molecule [10, 28]. Indeed, it is frequent to obtain non isomorphic trees for the same group of organisms using different molecules or functional domains of a molecule. In general, one must expect that, in spite of the "quasi-clockwise" evolutionary behavior of organisms [51], every clock will not indicate the same time [25, 36].

Other problems related to molecular phylogeny deal with the incompatibility between geologic data and those obtained with some molecular clocks [13, 19], the influence of the alignment procedure on the topology of the tree [32], the dependence on the compositional difference among sequences, mainly their G+C content [22, 42, 50], or the possibility of horizontal gene transfer inducing artefacts [41, 43].

A large controversy has also followed the use of paralogous genes as a way to root the universal tree of life [18, 26]. Several studies have revealed contradictions between protein trees and rRNA trees; or even among protein trees themselves. Thus, different families of paralogous genes may produce four alternative rootings for the universal tree and every possible grouping among the major lineages may appear [8, 16].

Presently, it is clear that not all the phylogenetic questions can be answered with such a simple method as sequence analysis. The study of biodiversity and evolutionary patterns of organisms

requires the complex combination of both phenotypic and genotypic information [3, 17, 21, 24]. The recent proposal of polyphasic taxonomy as a consensus for bacterial systematics also points in the same direction (reviewed in [45]).

During the last decade our group has developed a system to study the phylogeny of organisms based on the functional analysis of their protein synthesis machinery [2, 33, 40]. We have proposed the term "functiotype" for this singular part of the phenotype that comprises basic cellular functions like replication, transcription, translation or energy yielding processes [4, 6].The advantages of the translational apparatus over the other "functiotypic" ones are diverse. They are mainly based on the large amount of structural, functional and genotypic information available for the ribosomal systems. Indeed, protein synthesis is a basic and universal function of all organisms, and ribosomes are assemblies of a limited number (between 50 and 90) of genetically characterized macromolecules. These macromolecules, mainly the rRNAs, are the most traditionally accepted molecular clocks. They reveal the phylogenetic value of the ribosomal particle and its constituents. Moreover, their function does not depend on environmental effects.

The analysis of the ribosomal systems has been done using protein synthesis inhibitors with different domain and functional specificities [46]. The cell-free in vitro analysis performed avoids problems related to the transport, inactivation or pleiotropic effects of antibiotics. It also allows us to optimize the ionic conditions for every system, to make the necessary control experiments, and to adequately collect and standardize the obtained data [9, 39]. Our results have shown the intrinsic phylogenetic value of the sensitivity of ribosomes to protein synthesis inhibitors.

A new mathematical method is presented here to extract most of the information contained in the inhibition curves for thirty-five representative ribosomal systems belonging to the Archaea, Bacteria and Eukarya domains. The statistical procedure is based on the main components analysis and has allowed for the construction of phenograms which closely resemble those of 16/18S rRNA comparison [6]. The application of this approach to particular subsets of organisms and/or antibiotics has proved the validity of our functional method to study both macro- and mesophylogeny of organisms. It has also been possible to define a minimal group of fifteen protein synthesis inhibitors which are enough to cluster organisms belonging to any of the three domains.

Data collection

More than forty ribosomal systems belonging to organisms from the three major lineages have been analyzed. Some of them for which the inhibition studies had not been exhaustive were eliminated, resulting in a complete set of data for 35 organisms. The ribosomal systems used in this work are listed in Table 1.

Table 1 Ribosomal systems tested in this functional analysis

Lineage and organism	Strain	Abbreviation
Archaea		
Haloarchaea		
Haloarcula californiae	ATCC 33799	*H cali*
Haloarcula sinaiiensis	ATCC 33800	*H sina*
Halobacterium halobium	ATCC 43241	*H halo*
Halobacterium marismortui	ATCC 43049	*H mari*
Halobacterium salinarium	ATCC 33171	*H sali*
Halococcus morrhuae	ATCC 17082	*H morr*
Haloferax gibbonsii	ATCC 33959	*H gibb*
Haloferax mediterranei	ATCC 33500	*H medi*
Natronobacterium pharaonis	ATCC 43100	*N phar*
Natrononococcus occultus	ATCC 43101	*N occu*
Sulfur-dependent thermophiles		
Acidianus brierleyi	DSM 1651	*A brie*
Acidianus infernus	DSM 3191	*A infe*
Desulfurococcus mobilis	DSM 2161	*D mobi*
Metalosphera sedula	DSM 5348	*M sedu*
Sulfolobus solfataricus	DSM 1616	*S solf*
Thermococcus celer	DSM 2476	*T cele*
Thermoplasma acidophilum	DSM 1728	*T acid*
Thermoproteus tenax	DSM 2078	*T tena*
Methanogens		
Methanobacterium formicicum	DSM 1535	*M form*
Methanobacterium thermoautotrophicum	DSM 1053	*M ther*
Methanococcus vannielii	DSM 1224	*M vann*
Bacteria		
Cyanobacteria		
Anabaena sp.	ATCC 29151	*A sp.*
Prochlorothrix hollandica	ACC 15–2	*P holl*
Synechococcus sp.	PCC 7942	*S sp.*
Chloroplast from *Spinacia oleracea*		*Ch S o*
Proteobacteria		
Chromatium vinosum	ATCC 17899	*C vino*
Escherichia coli	ATCC 29417	*E coli*
Rhodobacter sphaeroides	ATCC 17023	*R spha*
Vibrio costicola	ATCC 33508	*V cost*
Eukarya		
Chlamydomonas reinhardtii	SAG 11–32b	*C rein*
Neurospora crassa	ATCC 24698	*N cras*
Rattus sp.		*R sp.*
Saccharomyces cerevisiae	Y 166	*S cere*
Tetrahymena thermophila	ATTCC 30008	*T ther*
Triticum aestivum		*T aest*

The different ribosomal systems have been tested in optimized in vitro translation systems against 38 protein synthesis inhibitors belonging to the three groups of specificity: I (inhibitors of bacterial ribosomes), II (inhibitors of eukaryotic ribosomes) and III (universal inhibitors). It must be noted that this classification of antibiotics [46] was previous to the establishment of Archaea as the third domain [49]. Unfortunately, there has not been any protein synthesis inhibitor characterized up to now which is specific for Archaea. On the other hand, from a functional point of view, every antibiotic acts on one

of the three steps of the elongation process: ternary complex formation (A), peptidyl-transferase (B) or translocation (C). The antibiotics, their domain and functional specificities, and the major structural families to which they belong, are shown in Table 2.

Table 2 Protein-synthesis inhibitors tested in the present study. Functional specificities are abbreviated as A, B, and C (see text for details). Fifteen antibiotics selected as the minimal subset for developing functional clustering are marked with an asterisk

Antibiotic	Functional specificity	Structural family
Group I		
Althiomycin*	B	
Carbomycin-A*	B	Macrolide of 16 C atoms
Gentamycin	A	Aminoglycoside (Ag)
Griseoviridin*	B	
Kanamycin	A	Ag
Neamine	A	Ag
Neomycin	A	Ag
Paromomycin	A	Ag
Ribostamycin	A	Ag
Streptomycin	A	Ag
Thiostrepton*	A	
Tobramycin	A	Ag
Tylosin	B	Macrolide of 16 C atoms
Viomycin*	C	
Virginiamycin-M	B	
Group II		
Alpha-sarcin*	A	Protein (16.8 kDa)
Anisomycin*	B	
Cryptopleurine*	C	Tilophora alkaloid
Cycloheximide*	C	Glutarimide
Haemanthamine	B	Narciclasine
Harringtonine	A + B	
Mitogillin	A	Protein (16.2 kDa)
Narciclasine	B	Narciclasine
Restrictocin	A	Protein (16.3 kDa)
Streptimidone	C	Glutarimide
Streptovitacin-A	C	Glutarimide
Toxin-T2	B	Tricotecene
Tubulosine*	C	
Tylophorine	C	Tilophora alkaloid
Group III		
Amicetin	B	Citosine analog
Anthelmycin*	B	Citosine analog
Blasticidin-S	B	Citosine analog
Edeine-A1*	B	
Fusidic acid*	A	
Hygromycin-B	A + C	Aminoglycoside analog
Puromycin	B	
Sparsomycin*	B	
Tetracycline*	A	

The preparation of the different cell-free systems and the conditions for the cell-free protein synthesis assays are described in the following references [1, 2, 9, 37, 38, 40; Casquero and Amils, unpublished results].

Preliminary analysis: estimation of the fractal dimension There are, in general, D = 5 measurements for each organism–antibiotic pair. They correspond to concentrations of the antibiotic in the in vitro translation sample which are 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M. Thus, we have built a complex databank which has the advantage of covering an extensive set of organisms and inhibitors at different concentrations. Nevertheless, we face the disadvantage of having to process a huge amount of numerical information to extract the phylogenetic value from this inhibition data. In previous treatments of our results [4] some preliminary methods were used to analyze them. Although they showed interesting correlations, an oversimplification of the data was produced, and therefore a significant part of the functional information was not considered. Another problem recently solved was the original bias of our data towards the archaeal systems, which might have affected the accuracy of the analysis.

In general we assume that all variables have a gaussian distribution; and that errors in separate measurements are uncorrelated. Since computer time is proportional to K^D, it is advisable to decrease the dimension of the original data set, preserving its informational content. The problem in estimating the D' dimension of an arbitrary figure has been deeply investigated in the theory of fractal geometry [5, 44]. Its application to our problem has shown that it is possible to diminish the original dimension from D = 5 to D' = 2 without decreasing significantly the information about the process (Koroutchev et al. 1998, in preparation).

Principal components analysis Once D' = 2 is determined for our set of data, it is possible to consider two principal vectors v_1 and v_2 to describe the system. The average relative error in the distances results in less than the 5% with this simplification. This means that it in the next steps of our analysis it is permissible to use every pair of antibiotic–organism for which there are at least two inhibition values available.

Cluster analysis The space defined by the two main vectors has been used for determining the coordinates of the organisms under study. This representation allows us to make a cluster analysis of the organisms, based on the nearest neighbor approach [29, 41].

Estimation of errors We assume that our data are subject to different type of errors, and, therefore, the distances are not strictly ultrametric. Branching errors will then appear in the final dendrograms, showing the branching interval associated to every grouping. It has been proved that general branching errors do not affect the topology of the different clusters. The estimation of the errors associated to the statistical treatment of the data constitutes another important improvement of the present method with respect to previous analysis [2, 41].

Interpretation of missing organism–antibiotic pairs Some considerations must be made about the pairs organism–antibiotic for which the inhibition study was not performed. It must be noted that, in spite of the exhaustive experimental analysis developed, not all the combinations were available. The main example of

this limitation is the impossibility of using aminoglycoside antibiotics against extreme halophilic archaea. Since there is a high concentration of cations required for the maintenance of the functional structure of halophilic ribosomes, a competition occurs for the binding sites between the cationic inhibitors and the fundamental cations [4, 39]. A way to overcome this problem when analizing the data was necessary. The easiest one was to ignore all ribosomes and antibiotics for which not every pair existed. Nevertheless, we would have lost a significant part of the experimental information (more than 25%). Therefore, we have defined the distance between two cellular systems by calculating it from the maximal subset of common antibiotics used with both of them. This is a good way for using all the information available at each stage.

Sequence comparison of the organisms

The method described above has allowed us to cluster the organisms under study. It is possible to use all the inhibitors in the mathematical analysis, or to select a particular group of them to make the clustering of some organisms. A premise of the method is that the number of independent variables used (antibiotics) must be greater than the taxonomic units to classify (organisms). This has a particular importance when using a subset of antibiotics for classifying a group of organisms. In any case, an interesting consequence is that it is possible to eliminate either some of the inhibitors or organisms of the analysis without disturbing the topology of the resulting phenograms. This clearly indicates the consistency of our data.

In order to prove the correlation between our results and the clustering obtained with the analysis of the small ribosomal subunit (SSU) rRNA, we have performed a parallel sequence comparison of the organisms under study. The programs GROWTREE [11], CLUSTAL V [23] and PHYLIP [15] have been used to generate phenograms and phylogenetic trees of the organisms. In every case there was a concordance of our functional clustering and that obtained with rRNA. This proves the phylogenetic value of our analysis. Figure 1 shows one of the dendrograms obtained with our functional analysis (left) and with the 16/18S rRNA sequence comparison (right) for a representative number of translational systems. We have selected 22 from the total set of 35 organisms in order to have a homogeneous representation of the three domains. All antibiotics listed in Table 2 were considered in this analysis. As described, the functional dendrogram shows the associated errors as crosses above each branching point.

The overall topology of both dendrograms results very close, revealing a clear separation among the three domains. The functional phenogram does not have a root and it shares the typical major branching order of the clusterings according to ribosomal RNA: Archaea appearing closer to Bacteria than to Eukarya [35]. Within Eukarya, the presence of the ciliate *Tetrahymena thermophila* as an outgroup of the other organisms is very interesting, because they cluster in a different way in both dendrograms.

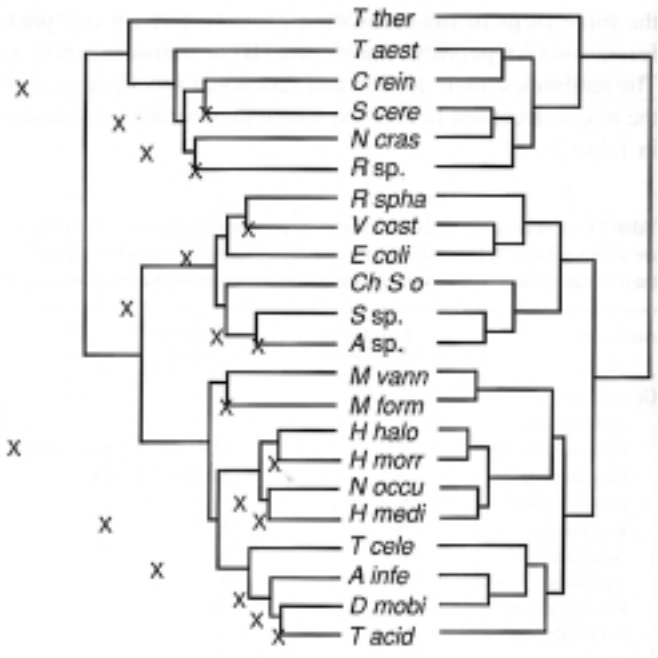

Fig. 1 Dendrogram obtained with the present functional analysis (left) and 16/18S rRNA sequence comparison (right). Abbreviations of organisms are shown in Table 1. Branching errors are marked in the functional phenogram

In Bacteria, a clear separation is observed between Proteobacteria and Cyanobacteria, although the internal branching order of the first clade depends on the system used. The inclusion of the *Spinacia oleracea* chloroplast into the cyanobacterial cluster clearly indicates that the ribosomes of the chloroplast mantain the sensitivity pattern of the bacterial group from which they originated. Thus, the endosymbiotic origin of organella [20, 30] is also reflected from a functional point of view.

Among Archaea, methanogens, extreme halophiles and sulfur-dependent thermophiles are classified in three separate clades as it occurs in the rRNA dendrogram. The established relation between methanogens and extreme halophiles [34, 50] is not observed in the functional phenogram, although in fact our analysis does not clearly define the branching order among the three archaebacterial groups. Halophiles show a very similar branching topology in both dendrograms. Indeed, it has been the group with the closest branching order for both phylogenetic systems in every analysis performed. This illustrates that the high sequence similarity displayed by their rRNAs [27] is functionally correlated. However, within sulfur-dependent thermophiles a similar pattern does not appear in both dendrograms. This can be related to the functional particularities exhibited by hyperthermophilic archaeal ribosomes, which have been characterized as the most refractory group to the protein synthesis inhibitors described so far [40].

As the next step of our functional analysis, we have studied the possibility of defining a minimal set of antibiotics which may be used for clustering the organisms. This simplification

has the advantage of eliminating some antibiotics whose specificity or mechanism of action are very similar [46] and therefore do not increase significantly the functional information or our data. Fifteen antibiotics (see Table 2) have been selected to homogeneously represent the whole set of them. Aminoglicosides have been excluded because their use is limited to non-halophilic organisms [4, 39].

Figure 2 shows the functional phenogram obtained with this minimal set of antibiotics when six representative organisms are analyzed. The overall topology of the universal tree showed in Fig. 1 is mantained, and the separation in three domains does not depend on the combination of the chosen organisms. Functional phenograms consistent with SSU rRNA secuence comparison have been also obtained with a selection of organisms belonging to Eukarya, Bacteria or Archaea. This reveals the feasibility of the method for studying phylogeny at different levels of taxonomic diversity.

The strong correlation obtained between our functional analysis and the sequence comparison clearly shows the value of the inhibition data to study macro- and mesophylogeny of organisms. To understand the basis of this interesting relationship it must be pointed out that the most informative parts of the SSU rRNA sequences are assumed to be the very conserved, taxonomically consistent, unpaired regions [12]. In spite of the limitation in the number of sequences available, this is also true for the large ribosomal subunit (LSU) rRNA molecules, whose phylogenetic analysis generates dendrograms and trees similar to those of the 16/18S rRNA [10]. It has been proved that some of these loop characters are associated with functional sites [14], although not much is known about how these regions are spatially disposed, or how tertiary and quaternary structures are established within the ribosome.

Phylogenetic value of ribosomal sensitivity to antibiotics

The antibiotics are small molecules in comparison to the ribosomal particle, and they act as specific functional effectors for the protein synthesis process. With the analysis of point mutations in the rRNA conferring resistance to some inhibitors, and the application of footprinting techniques to reveal the interaction sites of antibiotics, most of these target sites have been localized in the highly conserved regions of the rRNA. Furthermore, it is reasonable to assume that the ribosomal "functional space" with which antibiotics interact is not only dependent on these sequences. The three-dimensional contacts among such "functional loops" and other regions of the rRNAs, and also with some ribosomal proteins, will constitute the interaction sites of the specific inhibitors. Indeed, the antibiotics are acting as functional markers of the quaternary structure of the ribosomes. The phylogenetically consistent differences in the observed inhibition patterns reflect the evolution of the ribosomal particle, which is closely related to the progressive divergence of the sequences of its constituent macromolecules.

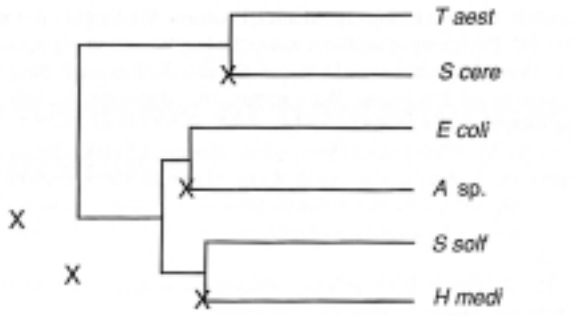

Fig. 2 Functional phenogram obtained from the data of six representative organisms and fifteen non redundant protein synthesis inhibitors (see Tables 1 and 2)

The existence of antibiotic-binding sites maintained in all ribosomal systems suggest that the basic components of the translational machinery have been preserved throughout evolution. In general, phylogenetically shared sensitivities should antedate the radiation of the three lineages. The progressive structural evolution of the ribosome would have promoted the appearance and loss of interaction sites for other effectors in different evolutionary lines. The present-day spectrum of sensitivities reflect the result of a "fine tuning" of the ribosomal function in different organisms, and therefore constitutes a record of their evolutionary history.

In conclusion, during the last decade, a number of authors [8, 16, 51] have clearly showed the limitations of molecular phylogeny techniques and have underlined the possibility of constructing wrong (although statistically robust) trees using only molecular data. As they have pointed, it is clear that the comparison of sequences may only be used for reconstructing the evolution of genes *but not of organisms*, and it is required the development of "new evolutionary paradigms where genomes, biochemistry and organisms are all considered in concert" [8]. Here we have shown that the intersection between phenotype and genotype is very valuable in the context of the translational apparatus. The structural-functional information provided by our analysis exploits that relationship and constitutes an interesting tool for the study of the evolution of organisms.

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