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Inferring the Phylogeny of Archaebacteria: The Use of Ribosomal Sensitivity to Protein-Synthesis Inhibitors

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Summary. The sensitivities to 37 protein-synthesis inhibitors were determined in cell-free translational systems for 13 Archaebacteria species. Multivariate factor analysis by principal components, cluster analysis by the unweighted pair-group clustering algorithm, and compatibility and parsimony methods of constructing phylogenetic trees were used both to infer the genealogical relationships within archaebacteria and to establish their relations with the eubacterial and eukaryotic kingdoms.

Key words: Archaebacteria — Protein-synthesis inhibition — Phylogeny

Introduction

Until very recently the classification of cell organization was dichotomous. On the basis of several structural and molecular features, cells were considered prokaryotic or eukaryotic. In the last years this view has been supplemented by a third kingdom, the Archaebacteria. Although there is a superficial resemblance between them, the archaebacteria are sufficiently distinct from the eubacteria in phenotype to justify a separation of the two at the highest taxonomic level (Woese et al. 1978). Archaebacterial microorganisms are prokaryotic in that they lack a nucleus and their external appearance is very similar to that of eubacteria, but their RNA polymerase, the nucleosomelike organization of their DNA, the presence of introns, and other features make them similar to the eukaryotic cells. Also, the structures of their membranes, their ATPases, and other macromolecular structures differ from those of typical prokaryotes and eukaryotes. Phylogenetically they also seem to constitute a new kingdom, one apparently as ancient as the other two (Woese and Fox 1977). Archaebacteria include three classes of microorganisms: the methanogens (MG), the extreme halophiles (HL), and the sulfur-metabolizing thermophilic archaebacteria (TH).

Several attempts have been made to clarify the phylogenetic relationships among the archaebacteria. Although some authors have used cell wall structure (Kandler 1982) or RNA polymerase subunit structures (Zillig et al. 1982), most of the studies have focused on several ribosomal components, especially different types of rRNAs. The comparison of catalogues of oligonucleotides generated by partial digestion of 16S rRNAs was the first method used to reveal the genealogical relationships among bacteria (Fox et al. 1980). The sequences of 5S rRNAs have also been used to generate archaebacterial dendrograms and to establish the relation of the archaebacteria to the other primary kingdoms (Fox et al. 1982; Hori et al. 1982), although the small size of the molecule (120 nucleotides) has given rise to doubts about its adequacy as a phylogenetic marker (Woese 1982). DNA–rRNA hybridization has also been used (Tu et al. 1982).

The three-dimensional structure of informational macromolecules can be also used in inferring phylogenies. Pieler et al. (1982) have reported on phylogenetic diversity among the three-dimensional

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structures of 5S rRNAs. Lake et al. (1984) used overall ribosome morphology, as revealed by electron microscopy, to trace the phylogenetic relationships among archaebacteria and the other primary kingdoms. The inhibitory actions of many antibiotics on protein synthesis depend on their interaction with specific target sites situated on the ribosome. Thus, different spatial configurations of the ribosome particle will presumably affect this interaction. If so, significant changes in ribosome quaternary structure can be reflected in different patterns of sensitivity to different specific inhibitors of the translational apparatus. From work with *Escherichia coli,* yeast mitochondria, and mammalian ribosomes, it is known that certain structural domains of large-ribosomal-subunit RNAs are involved in the interaction of translational inhibitors with the ribosome (see Noller 1984 for a review). Sensitivity or resistance to an antibiotic may therefore reside in some primary- or secondary-structural property of the rRNA. Since the specificity of the interaction between chloramphenicol and the eubacterial ribosome was first observed (Vazquez 1964), a comprehensive study has been made of the specificity of many translation inhibitors (Vazquez 1979). Because of their specificity, the antibiotics inhibiting the translational apparatus are considered good phylogenetic markers; thus, the sensitivity of chloroplast and mitochondrial ribosomes to eubacterial targeted antibiotics and their insensitivity to eukaryotic inhibitors have been considered to support strongly a eubacterial origin of the eukaryotic organelles (Chua and Luck 1974).

Several comparative studies on archaebacterial sensitivity to different antibiotics in vivo have been done (Hammes et al. 1979; Hilpert et al. 1981; Pecher and Böck 1981). However, for phylogenetic purposes it is advisable to use antibiotic patterns determined in cell-free systems, where problems of permeability or antibiotic modifications can be overcome. We selected the patterns of sensitivity (determined in cell-free systems) to 37 translational inhibitors of 13 representative archaebacteria for study. Multivariate factor techniques, cluster analysis, and compatibility and parsimony methods of constructing phylogenetic trees were used both to infer the genealogical relationships within the Archaebacteria and to establish the group's relations with the eubacterial and eukaryotic kingdoms.

Materials and Methods

Thirteen species representing the three archaebacterial groups were analyzed; they are listed in a footnote to Table 1. Their sensitivities to a representative group of antibiotics inhibiting ribosomal protein synthesis (also listed in the legend of Table 1) were determined in cell-free translational systems as described by Amils and Sanz (1986).

Three main statistical approaches were used to determine the phenetic and phylogenetic relationships: Factor analysis by principal components (Lebart and Fénelon 1975), the construction of a phenogram by the unweighted pair-group clustering method (UPGMA; Sokal and Sneath 1963), and the estimation of a phylogenetic tree. The last step was carried out using both the conventional Wagner procedure aiming to minimize the homoplasy (Eck and Dayhoff 1966; Kluge and Farris 1969) and a compatibility method (Le Quesne 1969). The following computer programs were used: COMPP (Lebart and Fénelon 1975), BMDP2M from the BMDP package (Dixon and Brown 1979), WAGPROC (Swofford 1981), and CLIQUE from the PHYLIP package (Felsenstein 1982). These programs were run on VAX 11/750 or IBM 4341 machines.

Results

The patterns of sensitivity of 13 archaebacterial species to 44 representative protein-synthesis inhibitors, as determined in cell-free systems, were compiled from several sources (Amils and Sanz 1986; Böck et al. 1985; Cammarano et al. 1985; R. Amils and J.L. Sanz, unpublished). The patterns are shown in Table 1. For comparison, we also compiled data on the eubacterium *E. coli* and the eukaryote *Saccharomyces cerevisiae.* Since the insensitivity of the halobacterial ribosomes to aminoglycoside antibiotics can be due to competition between the highly concentrated monovalent cations and the antibiotic and not to a lack of binding sites on the ribosomes, a total of eight aminoglycosides were not taken into account in the present study. For the remaining 37 antibiotics, sensitivity (as defined in Amils and Sanz 1986) of one species to a particular inhibitor, which means presence of the corresponding target site on the ribosome, was scored as 1, and

Asterisks on last two lines indicate characters forming the two largest cliques (see text). Species: Ss, *Sulfolobus solfataricus*; Ta, *Thermoplasma acidophila*; Ds, *Desulfurococcus mobilis*; Tc, *Thermococcus celer*; Tp, *Thermoproteus tenax*; Mf, *Methanobacterium formicicum*; Mt, *Methanobacterium thermoautotrophicum*; Mv, *Methanococcus vannielii*; Hd, *Halobacterium mediterranei*; Hr, *Halobacterium marismortui*; Hi, *Haloarcula sinaiiensis*; Ha, *Halobacterium salinarium*; Hg, *Halobacterium gibbonsii*; Ec, *Escherichia coli*; Sc, *Saccharomyces cerevisiae.* Antibiotics: THI, thiostrepton; ANI, anisomycin; KIR, kirromycin; TT2, toxin-T2; CAR, carbomycin-A; GRI, griseoviridin; PUR, puromycin; VIM, virginiamycin-M; SPC, spectinomycin; TET, tetracycline; VIR, viridogrisein; MIT, mitogillin; TUB, tubulosine; SAR, alpha-sarcin; HAR, harringtonine; HHA, homoharringtonine; PUL, pulvomycin; BRU, bruceantin; HAE, haemantamine; NAR, narciclasine; PRE, pretazetine; ALT, althiomycin; CHX, cycloheximide; CRI, cryptopleurine; STR, streptimidone; STP, streptovitacin-A; RES, restrictocine; TYT, tylophorine; FUS, fusidic acid; VIO, viomycin; HYB, hygromycin-B; AMI, amicetin; ANT, anthelmycin; BLA, blasticidin-S; TYL, tylosin; SPA, sparsomycin; EDE, edeine-A1

Table 1. Sensitivity to protein-synthesis inhibitors in 13 archaebacterial species, the eubacteria *Escherichia coli,* and the eukaryote *Saccharomyces cerevisiae*

	1 THI	2 ANI	3 KIR	4 TT2	5 CAR	6 GRI	7 PUR	8 VIM	9 SPC	10 TET	11 VIR	12 MIT	13 TUB
Ss	0	0	0	0	0	0	1	0	0	1	0	0	0
Ta	1	0	0	0	0	0	1	1	0	1	0	1	0
Ds	1	0	0	0	0	0	1	1	0	0	0	1	0
Tc	1	0	0	0	0	0	1	0	0	1	0	1	0
Tp	0	0	0	0	0	0	1	0	0	1	0	1	0
Mf	1	1	1	0	1	1	1	1	1	1	0	1	0
Mt	1	1	0	0	1	0	1	1	0	1	0	1	0
Mv	1	0	0	0	1	0	1	1	0	1	0	1	0
Hd	1	1	0	0	1	0	1	0	0	1	1	0	0
Hr	1	1	0	0	1	1	1	0	0	1	0	0	0
Hi	1	1	0	0	1	1	1	1	0	1	1	0	0
Ha	1	1	0	0	1	1	1	1	0	1	1	0	0
Hg	1	1	0	0	1	1	1	1	0	1	1	0	1
Ec	1	0	1	0	1	1	1	1	1	1	1	0	0
Sc	0	1	0	1	0	0	1	0	0	1	0	1	1
Cq. 1			*	*	*	*	*		*	*			
Cq. 2		*	*	*	*	*	*		*	*			

	14 SAR	15 HAR	16 HHA	17 PUL	18 BRU	19 HAE	20 NAR	21 PRE	22 ALT	23 CHX	24 CRI	25 STR	26 STP
Ss	1	0	0	0	0	0	0	0	0	0	0	0	0
Ta	1	0	0	0	0	0	1	0	0	0	0	0	0
Ds	1	0	0	0	0	0	1	0	0	0	1	0	0
Tc	0	0	0	0	0	0	1	0	0	0	0	0	0
Tp	1	0	0	0	0	0	0	0	0	0	0	0	0
Mf	1	0	0	1	1	0	1	0	1	0	1	0	0
Mt	1	0	0	1	1	0	1	0	1	0	1	0	0
Mv	1	0	0	1	1	0	0	0	1	0	0	0	0
Hd	0	0	0	1	0	0	1	0	0	0	1	0	0
Hr	0	0	0	1	0	1	1	0	1	0	1	0	0
Hi	0	0	0	1	1	0	1	0	1	0	0	0	1
Ha	0	1	0	1	0	0	1	0	1	0	0	1	0
Hg	0	0	0	1	0	1	1	0	1	0	1	0	0
Ec	0	0	0	1	0	0	0	0	1	0	0	0	0
Sc	1	1	1	0	1	1	1	1	0	1	1	1	1
Cq. 1			*	*				*	*	*			
Cq. 2			*	*				*		*			

	27 RES	28 TYT	29 FUS	30 VIO	31 HYB	32 AMI	33 ANT	34 BLA	35 TYL	36 SPA	37 EDE
Ss	0	0	0	0	0	0	0	0	0	1	0
Ta	1	0	0	1	1	0	1	0	0	1	1
Ds	1	0	0	0	0	0	0	0	0	0	1
Tc	1	0	0	0	0	0	0	0	0	0	1
Tp	1	0	1	1	1	1	1	0	0	1	1
Mf	1	1	1	1	1	0	1	1	1	1	1
Mt	1	1	1	1	1	0	1	1	1	1	1
Mv	1	0	1	1	0	0	1	1	0	1	0
Hd	0	0	1	0	1	1	1	1	1	1	0
Hr	0	0	1	0	1	1	1	1	1	1	0
Hi	0	0	1	0	1	1	1	1	1	1	1
Ha	0	0	1	1	1	1	1	1	1	1	1
Hg	0	0	1	0	1	1	1	1	1	1	1
Ec	0	0	1	0	1	1	1	1	1	1	1
Sc	1	1	1	0	1	1	1	1	0	1	1
Cq. 1			*				*	*		*	
Cq. 2			*				*	*		*	

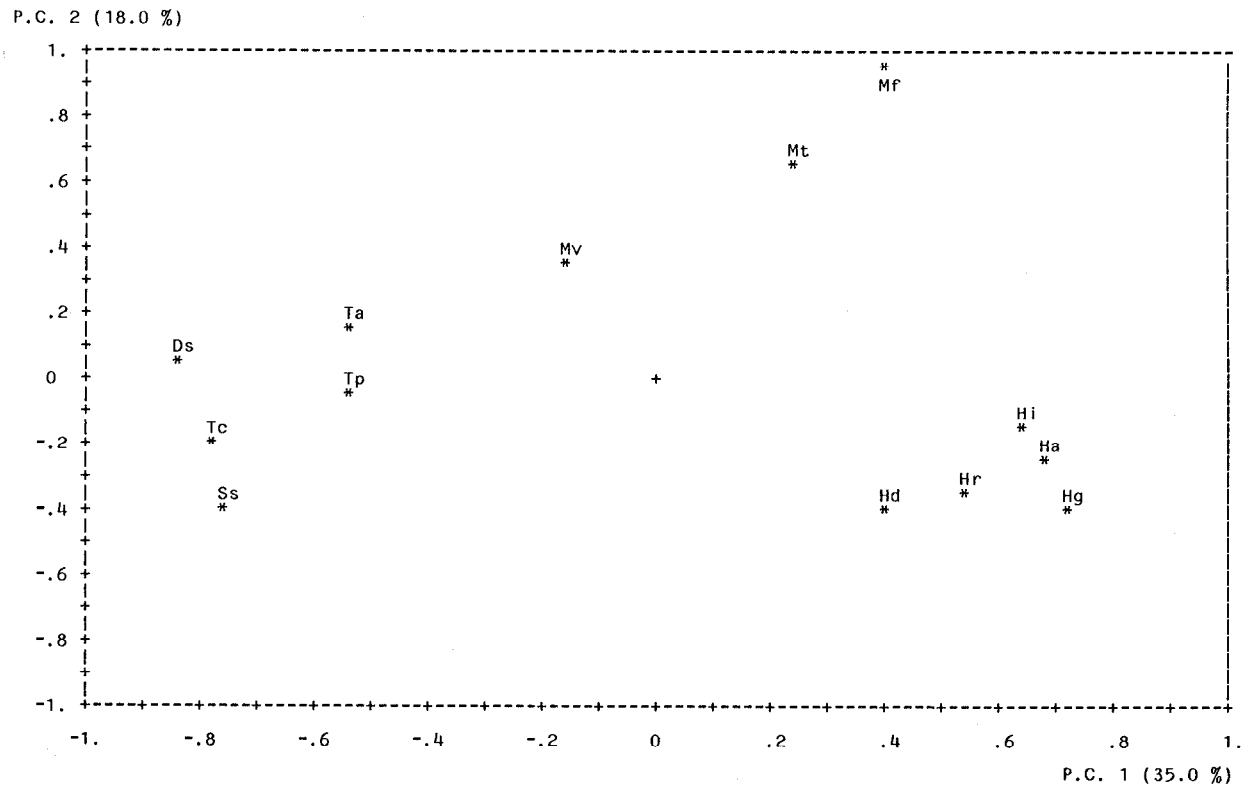

Fig. 1. Ordination obtained by principal-component analysis. The plane is defined by factors PC1 and PC2. Abbreviations as in Table 1

insensitivity, which means lack of the corresponding binding site, as 0. Thus, a 37 × 15 data matrix representing the distribution of character states over the 15 species was obtained (Table 1). Since the antibiotic inhibition curves have different slopes and shapes, other scoring methods with larger ranges (0–4, 0–5) were tried, but did not give significantly different results. However, they were discarded because their usefulness seems to be limited to very closely related species for the following reasons: (1) It is not certain that an unequivocal correspondence exists between the assigned scores and ribosome structural features; (2) since the determinations of antibiotic sensitivities in cell-free systems have been carried out under very different physicochemical conditions, a true standardization of the scores is not easily established.

A first picture of the phenetic relationships among the archaebacterial species can be obtained through factorial and cluster analysis. Through principal-component analysis a two-dimensional ordination was obtained (Fig. 1). The plane defined by principal components PC1 and PC2 explains 53% of the total variance contained in the data matrix. Good groupings were obtained for HL and TH species along the first axes, while the MG species are spread along intermediate positions.

With cluster analysis a 15 × 15 dissimilarity matrix was obtained. The distance D(A,B) between two species A and B is defined as the sum of the absolute values of their differences in sensitivity. This distance has the form of a Manhattan metric:

$$D(A,B) = \sum |x(i,A) - x(i,B)|$$

The matrix of distances is presented in Table 2, and the phenogram obtained from this matrix by the UPGMA algorithm is shown in Fig. 2. It can be seen that the three main clusters correspond to the MG, HL, and TH species, respectively, in good agreement with the accepted taxonomy. Distances within the TH species range from 4.0 [between Ds and Tc (abbreviations as in Table 1)] to 11.0 (between Ds and Tp), with an average of 7.4. In the HL group, distances range from 4.0 (between Hr and Hd) to 8.0 (between Ha and the Hd–Hr grouping), averaging 5.4. Within the MG group the average distance is 6.6, with Mv far removed from both Mt and Mf. The average intergroup values of D are as follows: 17.36 between TH and HL, 14.4 between TH and MG, and 12.4 between HL and MG. This result agrees with data on crosshybridization of DNAs and 16S rRNAs (Tu et al. 1982).

The well-known connection between MG and HL species is clearly shown in the cluster. It is also apparent that *Methanococcus,* Mv, is split from the *Methanobacteria,* Mf and Mt, and more distant from the HL (14.2) than Mt, with a distance of 10.8, and Mf, with a distance of 12.2. Also, Mv is much closer

Table 2. Manhattan distances among species

	Ss	Ta	Ds	Tc	Tp	Mf	Mt	Mv	Hd	Hr	Hi	Ha	Hg	Ec	Sc
Ss	0.0														
Ta	9.00	0.0													
Ds	9.00	6.00	0.0												
Tc	7.00	6.00	4.00	0.0											
Tp	8.00	5.00	11.00	9.00	0.0										
Mf	22.00	13.00	17.00	19.00	16.00	0.0									
Mt	19.00	10.00	14.00	16.00	13.00	3.00	0.0								
Mv	12.00	9.00	13.00	13.00	10.00	10.00	7.00	0.0							
Hd	14.00	15.00	17.00	15.00	14.00	14.00	11.00	14.00	0.0						
Hr	16.00	17.00	19.00	17.00	16.00	12.00	11.00	14.00	4.00	0.0					
Hi	19.00	16.00	20.00	18.00	17.00	11.00	10.00	13.00	7.00	7.00	0.0				
Ha	20.00	15.00	21.00	19.00	16.00	12.00	11.00	14.00	8.00	8.00	5.00	0.0			
Hg	20.00	17.00	19.00	19.00	18.00	12.00	11.00	16.00	6.00	4.00	5.00	6.00	0.0		
Ec	18.00	15.00	21.00	19.00	14.00	10.00	13.00	12.00	10.00	10.00	7.00	6.00	8.00	0.0	
Sc	22.00	19.00	21.00	21.00	16.00	20.00	17.00	22.00	20.00	20.00	21.00	22.00	20.00	28.00	0.0

Species: Ss, *Sulfolobus solfataricus*; Ta, *Thermoplasma acidophila*; Ds, *Desulfurococcus mobilis*; Tc, *Thermococcus celer*; Tp, *Thermoproteus tenax*; Mf, *Methanobacterium formicicum*; Mt, *Methanobacterium thermoautotrophicum*; Mv, *Methanococcus vannielii*; Hd, *Halobacterium mediterranei*; Hr, *Halobacterium marismortui*; Hi, *Haloarcula sinaiiensis*; Ha, *Halobacterium salinarium*; Hg, *Halobacterium gibbonsii*; Ec, *Escherichia coli*; Sc, *Saccharomyces cerevisiae*

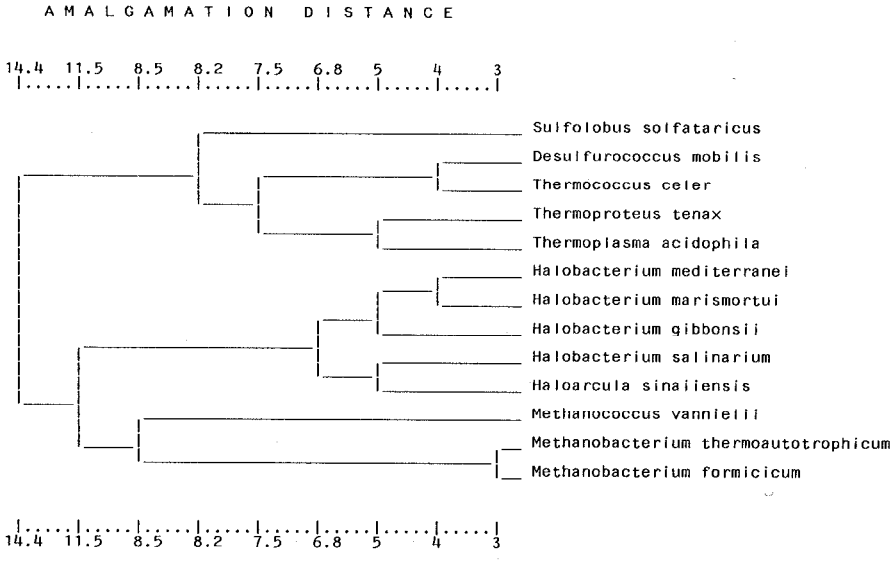

Fig. 2. UPGMA cluster for the Archaebacteria

to TH (averaging D = 11.4) than Mt (14.4) or Mf (17.4). The HL group is split in two subclusters (Hd–Hr–Hg and Ha–Hi), which are joined together at the lower intragroup amalgamation distance. Two clusters (Ds–Tc and Tp–Ta) constitute the core of TH; the very insensitive Ss is added last.

The phylogenetic relationships within archaebacteria and their connections with eubacteria and eukaryotes were inferred using two types of analysis: parsimony and compatibility. For a discussion of both methods see Felsenstein (1981, 1982). Both methods require that most characters have a low rate of change and that homoplasy (convergence) be rare. As mentioned above, this seems to be the case for ribosomal sensitivity to antibiotics. Since we do not know which state (sensitivity or insensitivity) is ancestral, and since reversion from a derived state to an ancestral one is possible (Böck et al. 1985), the Wagner parsimony method, which aims to minimize homoplasy, was used to obtain a first phylogenetic tree (Fig. 3). We also assayed the hypothesis that each character state evolved only once, which implies that one must search for the largest set of mutually compatible characters (called a clique). This can be done through a compatibility analysis. With this technique, two different cliques of 16 characters each, differing from one another by one character, were found (see Table 1). With these two sets of compatible characters we obtained very similar phylogenetic trees (Fig. 4).

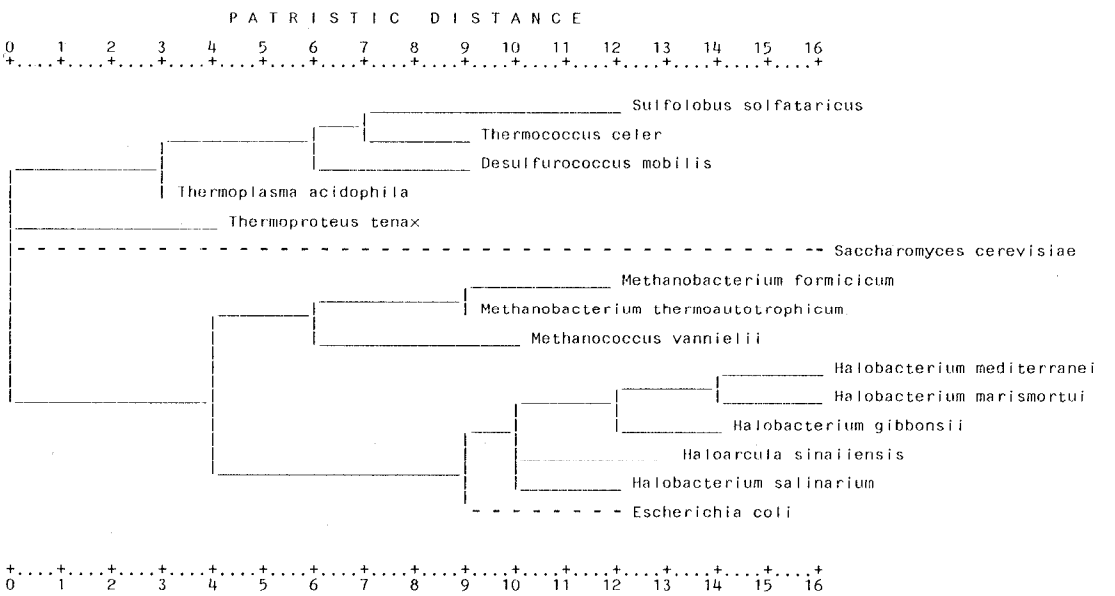

Fig. 3. Phylogenetic tree obtained by Wagner parsimony method rooted at midpoint of longest path and optimized for minimum homoplasy. The goodness-of-fit statistics are: total homoplasy, 348.0; deviation ratio, 0.241; index of consistency, 0.468; cophenetic correlation, 0.896; and total length of tree, 77

Discussion

It seems clear, from the agreement among the groupings obtained by all our statistical analyses and other groupings established by the molecular techniques mentioned above, that sensitivity to protein-synthesis inhibitors could be a useful tool for phylogenetic studies. Its validity perhaps would rest on a dependence of changes in sensitivity on changes in quaternary structure. The ribosomal particle is built from a direct DNA transcript (the rRNA) and ribosomal proteins, which act mostly as effectors regulating the particle function. Other points to consider in this respect are the basic functional importance of translational apparatus. It is also worth remarking that in contrast to rRNA sequence data, which consider all the changes, even those with no functional meaning, our approach takes into account only functionally significant changes, that is, those expressed at target sites for antibiotics.

One of the principal aims of this paper is to probe the phylogenetic relationships among archaebacteria, eubacteria, and eukaryotes; we selected *E. coli* and *S. cerevisiae* to represent these two last groups. We are aware that our results on the evolutionary relationships with the eubacterial and eukaryotic kingdoms can only be tentative. Work in progress in our laboratory with other eubacterial and eukaryotic species will allow us to corroborate these connections. Figure 5 is a representation of the relationships obtained by single linkage from the averaged distances among the different groups. It shows that *E. coli* clusters with HL, the average distance being 8.2. Average *E. coli* distances from MG and TH are greater (11.7 and 17.4, respectively). The eukaryotes are far from *E. coli* (D = 28), and their average distance from all archaebacteria is 20.07; they are fairly equidistant from all three archaebacterial groups (Sc–TH = 19.8; Sc–HL = 20.6; Sc–MG = 19.7). Thus, eukaryotes are much closer to archaebacteria than to eubacteria. This means that the translational apparatus of eukaryotes is more closely related to that of archaebacteria than to that of eubacteria.

These same connections are shown by the Wagner tree (Fig. 3). Three monophyletic groups can be seen in this tree: one including the MG and HL, which also includes the eubacterium *E. coli*; a second group corresponding to TH; and a third branch for the eukaryotes. Thus information provided by ribosomal inhibition supports the idea that that the Archaebacteria are a polyphyletic group. On taxonomic grounds, Tu et al. (1982) also claimed the existence of at least two clearly recognizable branches of the archaebacterial ur-kingdom. In their tree, the eubacteria share a nearby common ancestor with HL. The average phylogenetic distance of *E. coli* from HL is 8.2 and the average distance from MG is 14.3, while the distance from TH is greater, 19.4. This result supports the scheme that Böck et al. (1985) proposed based on several characters. The eukaryote *S. cerevisiae* shows its shortest phylogenetic distance with TH (the average distance is 23.4). This result is in good agreement with data on RNA polymerase homology (Zillig et al. 1982), ribosome morphology (Lake et al. 1984), and several other types of evidence (Böck et al. 1985). Furthermore, the closest organism to this eukaryote is the thermophile

Thermoplasma acidophila (patristic distance = 19.0); this finding could relate to the suggestion made by D. Searcy (1985) that *Thermoplasma* could be a close relative of the ancestral organism from which the eukaryotic cytoplasm was derived.

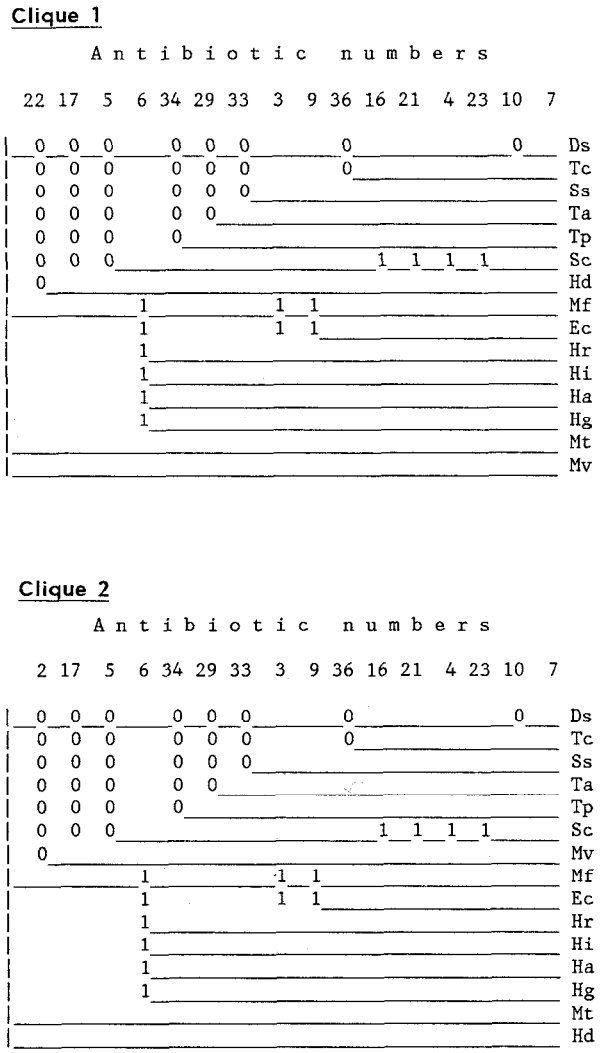

Fig. 4. Phylogenetic trees obtained by compatibility method. Insensitivity and sensitivity are indicated by 0 and 1, respectively. Numbers and abbreviations as in Table 1

Different functional domains of the ribosome seem to have evolved at different rates (Böck et al. 1985). Compatibility analysis allowed us to identify those antibiotic-binding sites with low evolutionary rates. A phylogeny relating distant species of archaebacteria, eubacteria, and eukaryotes was then inferred from these low-rate characters (Fig. 4). The principal branches in this phylogeny were in good agreement with both the phenetic relationships (Fig. 5) and the Wagner tree based on all the antibiotic sensitivities (Fig. 3).

On the basis of the drawn phylogenetic relationships, one can attempt to distinguish several classes of inhibitor sites on the ribosome:

1. Antibiotic binding sites with high evolutionary rates, which developed late in the evolution of the different lineages (all sites not included in either of the two cliques; see Table 1).
2. Binding sites with low evolutionary rates (all sites included in the two cliques; see Table 1). We can distinguish several subgroups:
   a. Sites maintained during the development of all (e.g., that for puromycin) or almost all (e.g., that for tetracycline) the archaebacterial, eubacterial, and eukaryotic ribosomes.
   b. Sites, such as those for pulvomycin, carbomycin-A, and blasticidin-S, present in neither eukaryotes nor thermophiles and acquired by a hypothetical common ancestor of eubacteria, HL, and MG (see Fig. 3).
   c. Sites acquired during the evolution of the eukaryotic (those for homoharringtonine, pretazetine, toxin-T2, and cycloheximide) or eubacterial (those for kirromycin and spectinomycin) lineages.

Perhaps a bias in our approach is that the struc-

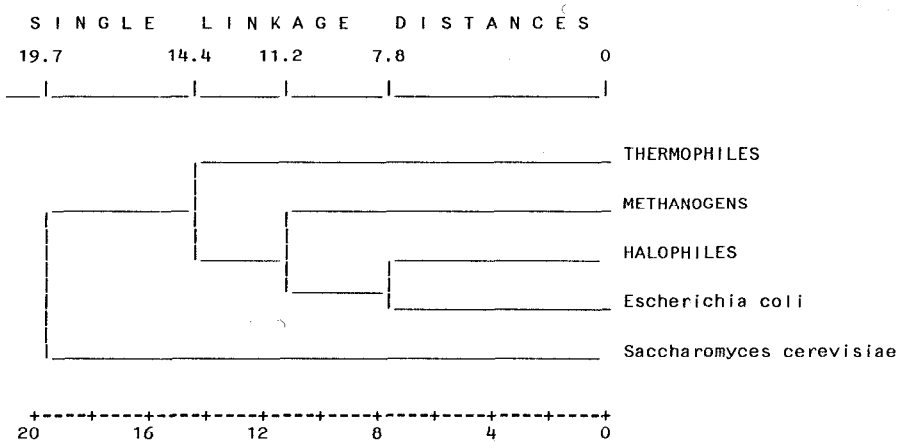

Fig. 5. Phenetic relationships among the archaebacterial groups, the eubacterium *Escherichia coli*, and the eukaryote *Saccharomyces cerevisiae*

tural markers used (antibiotics) were selected on the basis of their specificities, that is, their capacity to impair protein synthesis in eubacterial or eukaryotic ribosomes. It remains to be seen whether other antibiotics exist in nature that display a different specificity pattern (for instance, the ability to inhibit selectively archaebacterial protein synthesis), and if so, what the effects of the introduction of these data on our analysis will be. Of course, until such compounds are available, the approach used in this study seems to be the only appropriate one.

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