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Sequence analysis of twin ATP binding cassette proteins involved in translational control, antibiotic resistance, and ribonuclease L inhibition

Ian D. Kerr*

Centre for Biochemistry and Cell Biology, School of Biomedical Sciences, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, UK

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Abstract

Genome sequencing has identified open reading frames which belong to the ATP binding cassette (ABC) transporter family, but which are unlikely to be involved in transport phenomena. These frequently contain a pair of nucleotide binding domains (NBD) with no associated transmembrane domains. The functions of many of these twin-NBD proteins remain unknown. In this manuscript, sequence analysis has been employed to analyse two families of twin-NBD proteins, ABCE and ABCF. The ABCE proteins, postulated to be inhibitors of RNase L, are identified by two potential Fe–S metal-binding domains in addition to two NBDs. Surprisingly, ABCE homologues are identified in numerous species which apparently lack an RNase L, questioning the proposed function of these proteins. The ABCF proteins can be sub-divided into more than a dozen sub-classes. Intriguingly, sequence similarity is shown between eukaryotic ABCF proteins, which are involved in translation initiation and elongation, and prokaryotic ABCF proteins which are implicated in resistance to macrolide inhibitors of protein synthesis.

Keywords: GCN20; ABC50; Antibiotic resistance; Elongation factor; Nucleotide binding domain; Macrolide; Ribosome; Translation initiation; ATP binding cassette; Iron–sulphur

One of the largest groups of membrane proteins in most sequenced genomes is the ATP binding cassette (ABC) transporter family. The *Escherichia coli* genome contains 79 ABC proteins, occupying 5% of the entire genome [1]. In humans there are believed to be at least 48 ABC systems, responsible for, inter alia, antigen presentation, and insulin secretion. Defective human ABC transporters are implicated in diseases such as cystic fibrosis and adrenoleukodystrophy [2]. All ABC proteins are characterized by the presence of the eponymous cassette, otherwise known as the nucleotide binding domain (NBD), which contains a number of characteristic sequence motifs. In addition to the Walker-A and -B motifs identified in many families of ATPases, NBDs contain the ABC transporter signature motif (Sig), a conserved aromatic amino acid-containing region (Tyr-loop), a conserved glutamine loop (Gln-loop), and a conserved histidine loop (His-loop). These latter four sequence motifs distinguish ABC transporter NBDs from other ATPases [3]. In ABC proteins associated with membrane transport, the NBDs bind and hydrolyse nucleotide and transmit conformational changes to membrane spanning domains (MSDs), which typically form a pathway for the transported substrate. In most eukaryotic ABC transporters, the MSDs are fused to the NBDs in a single polypeptide, whilst in bacterial systems the NBDs and MSDs are frequently found localized together in operons [1].

Genome analysis has identified several sub-families of ABC transporters. In eukaryotes, the classification results in the designation of seven sub-families, ABCA to ABCG [2]. Five of these contain NBDs fused to MSDs and members of these groups are responsible for membrane transport processes. The remaining two groups, ABCE and ABCF, contain a pair of linked NBDs

* Fax: +44-115-970-9969.
E-mail address: ian.kerr@nottingham.ac.uk (I.D. Kerr).

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(NBD–NBD) which are not fused to MSDs. For example, in the yeast *Saccharomyces cerevisiae* the ABCE family contains a single member, in addition to six members of the ABCF family [4]. The functions of the ABCE and ABCF family are, therefore, unlikely to be transport-related. Bacterial and archaeal sequencing projects have also identified large numbers of twin-NBD proteins, which are not linked in operons to known transmembrane proteins [5]. In this article, the available sequence data for the eukaryotic ABCE and ABCF subfamilies of proteins, and for the non-transport-associated NBD–NBD open reading frames from bacteria have been analysed. This analysis suggests that a mechanistic similarity exists between eukaryotic members of the ABCF family involved in control of translation initiation and elongation, and that these proteins may also have functional similarity to prokaryotic ABCF proteins involved in mediating resistance to macrolide antibiotics.

Phylogenetic analysis

Sequence searching was performed using Psi-Blast, with default parameters. Sequences are referred to in figures and tables by GenBank [6] accession codes (a complete list of sequences employed is available from the author). Global pairwise and multiple sequence alignments were performed using AMPS [7] or ClustalW [8] employing the Blosum62 and Gonnet250 substitution matrices. For pairwise alignments 100 randomizations of each pair of sequences were performed to determine a similarity Z score. A Z score of >10 is equivalent to a p value of $<10^{-12}$ [9]. No manual manipulation of sequence alignments was performed. Phylogenetic trees were produced using Phylip (http://evolution.genetics.washington.edu/phylip.html) or GeneDoc (http://www.psc.edu/biomed/genedoc). Transmembrane (TM) helix prediction was carried out employing several algorithms, the complementarity and merits of which have recently been discussed [10]. Investigation of the presence of operons in archaeal and bacterial genomes was performed using the Genome Information Broker facility (website http://gib.genes.nig.ac.jp/main.php).

Interpretation of sequence analysis

Previously, proteins containing ABC transporter-like NBDs, but which have no transport function, have been demonstrated to be involved in nucleotide excision repair (UvrA [11]), DNA mismatch repair (MutS [12]), DNA double-strand break repair (Rad50 [13]), and chromosomal segregation (SMC [14]). All of these proteins contain a single NBD within the polypeptide (with the exception of UvrA which contains three linked NBDs), although dimerization is required for function in the cases of SMC, MutS, and Rad50 [15]. In the current manuscript the two clusters of twin NBD proteins of eukaryotic ABC transporters [2] are examined. Potential homologues of these proteins in archaea and eubacteria are identified, and hypotheses that may assist in the identification of their functional roles are considered.

Classification of ABCF sequences

The 810 amino acid sequence encoding human ABCF1 (ABC50) was employed as the Psi-Blast search query to identify other ABCF members. Sequence homologues in the ABCF family were identified in eubacteria, and other eukaryotes, although none was identified in any archaea. Searches of 25 archaeal translated genomic databases provided no NBD–NBD sequences with greater than 15% end-to-end identity to mammalian ABCF sequences. The apparent absence of ABCF proteins from archaea may be consistent with the hybrid nature of their translation initiation [16]. Among eukaryotes, *Arabidopsis thaliana* and *S. cerevisiae* displayed the greatest number of ABCF sequences with five genes apiece [4,17]. Two of the *A. thaliana* sequences satisfy criteria that suggest they have been acquired ancestrally from cyanobacteria [18]. Pairwise sequence alignments were employed to reduce the more than 500 retrieved sequences to a subset of 311. In particular, if open reading frames from two closely related bacterial species shared 95% amino acid sequence identity then one was excluded. None of the final 311 sequences correspond to an NBD–NBD protein that has been identified as the ATP-hydrolysing component of an ABC transporter, and none of the bacterial genes are linked directly in an operon to a gene encoding a multiple membrane spanning protein that could act as the MSD of an ABC transporter. Furthermore, no putative TM helices were consistently predicted in any of the NBD–NBD sequences. Thus, it seems likely that all of the identified NBD–NBD proteins have a non-membrane localization and are not involved in membrane transport phenomena.

Phylogenetic analysis of the ABCF candidate sequences enabled the identification of 13 sub-classes. In Table 1 these are presented with a brief annotation of protein function or localization, where established. To improve the clarity of the data, the phylogenetic analysis of eukaryotic and prokaryotic ABCF proteins is presented separately in Figs. 1 and 2. Four classes (I–IV) consist of eukaryotic proteins exclusively, while the other nine contain exclusively bacterial proteins (with the exception of two *Arabidopsis* genes acquired ancestrally in Class IX). The nine sub-classes of bacterial proteins contain all of the NBD–NBDs previously

Table 1
Sub-classification of ABCF sequences

	N	*L*	Example	Function	Reference
I	8	785	*H. sapiens* ABC50 GI:10947135	Promote association of aminoacyl-tRNA to eIF2	[21]
II	15	620	*H. sapiens* ABCF2 GI:10179834	Iron response factor? Yeast homologue is localized to cytoplasm	[39,40]
III	11	735	*S. cerevisiae* GCN20 GI:1169871	Activation of eIF2-kinase	[22]
IV	9	1090	*S. cerevisiae* EF3A GI:119180	Yeast translation factor and mRNA export	[25]
V	52	555	*E. coli* yjjK GI:2851615		[1]
VI	12	635	*S. aureus* mw0682 GI:21282411		[41]
VII	18	627	*E. coli* uup GI:2506112	RecA independent DNA excision	[20]
VIII	28	612	*B. subtilis* ydiF GI:3025120		[19]
IX	10	585	*A. thaliana* GI:15912315		[17]
X	43	530	*E. chrysanthemi* ybiT GI:15384866	Pathogen virulence	[42]
XI	49	630	*E. coli* yheS GI:1176239		[1]
XII	16	540	*S. fradiae* tlrC GI:135918	Macrolide resistance	[27]
XIII	13	540	*S. epidermidis* msrA GI:127359	Macrolide resistance	[30]

Roman numerals denote sub-class, N—number of proteins/open reading frames in the established sub-groups, L—average length of the members of each class. Each example is listed with its GenBank accession code and/or protein identification. The function is given, where known, together with an appropriate reference.

suggested in *E. coli* and *Bacillus subtilis* genomic analysis as non-transport ABC proteins [1,19]. The most well-characterized bacterial non-transport NBD–NBD protein is *E. coli* Uup, which has been identified by mutational analysis to be involved in the RecA-independent excision of transposable elements, and in the deletion of single copies of tandem chromosomal repeats [20] although there are no evident helicase motifs within the Uup sequence, suggesting interaction between Uup and other proteins. The uup gene is part of a conserved operon structure (in, for example, *Yersinia pestis* and *Salmonella enterica*) and is co-transcribed with an upstream gene (ycbY) [20]. The majority of the other bacterial ABCF sub-classes remain poorly characterized, with the exception of Classes XII and XIII which are discussed below.

Do eukaryotic ABCF proteins have a shared tRNA interaction mechanism?

All three of the eukaryotic ABCF classes identified by phylogenetic analysis (Fig. 1) for which there is functional information contain proteins involved in the control of protein translation. Class I proteins, exemplified by ABCF1 from *Homo sapiens* [21], interact with eIF2 and with 40S and 60S ribosomal subunits in an ATP-dependent manner. Functional investigation of the eIF2/ABC50 interaction suggests that association of methionyl-tRNA with eIF2 is significantly enhanced by ABC50. This interaction supports the hypothesis that Class I proteins control translation initiation at the level of tRNA-loading at the eIF2 ternary complex [21]. Class III proteins (exemplified by yeast GCN20) are also capable of ATP-dependent ribosomal interaction [22]. Interestingly, GCN20 associates with GCN1 to form a complex capable of stimulating eIF2-kinase (GCN2) [23]. This stimulation depends upon the loading of the kinase with an uncharged tRNA. The current sequence analysis predicts that Class III proteins are responsible for the interaction of the GCN2 with uncharged tRNA molecules, implying a functional similarity between Class III and Class I proteins. While translation *initiation* is influenced by Class I and Class III proteins, the proteins of Class IVA (yeast elongation factor 3) mediate control of translation *elongation*. The A (amino-acyl tRNA binding) and E (uncharged tRNA exit) sites of the yeast ribosome appear to be translationally linked such that occupancy of the A-site by aminoacylated-tRNA is coupled to removal of the deacylated-tRNA from the E-site [24,25]. The clustering of EF3 with other proteins involved in tRNA interactions with ribosomes prompts the hypothesis that EF3 may be responsible for an ATPase dependent removal of the deacylated-tRNA

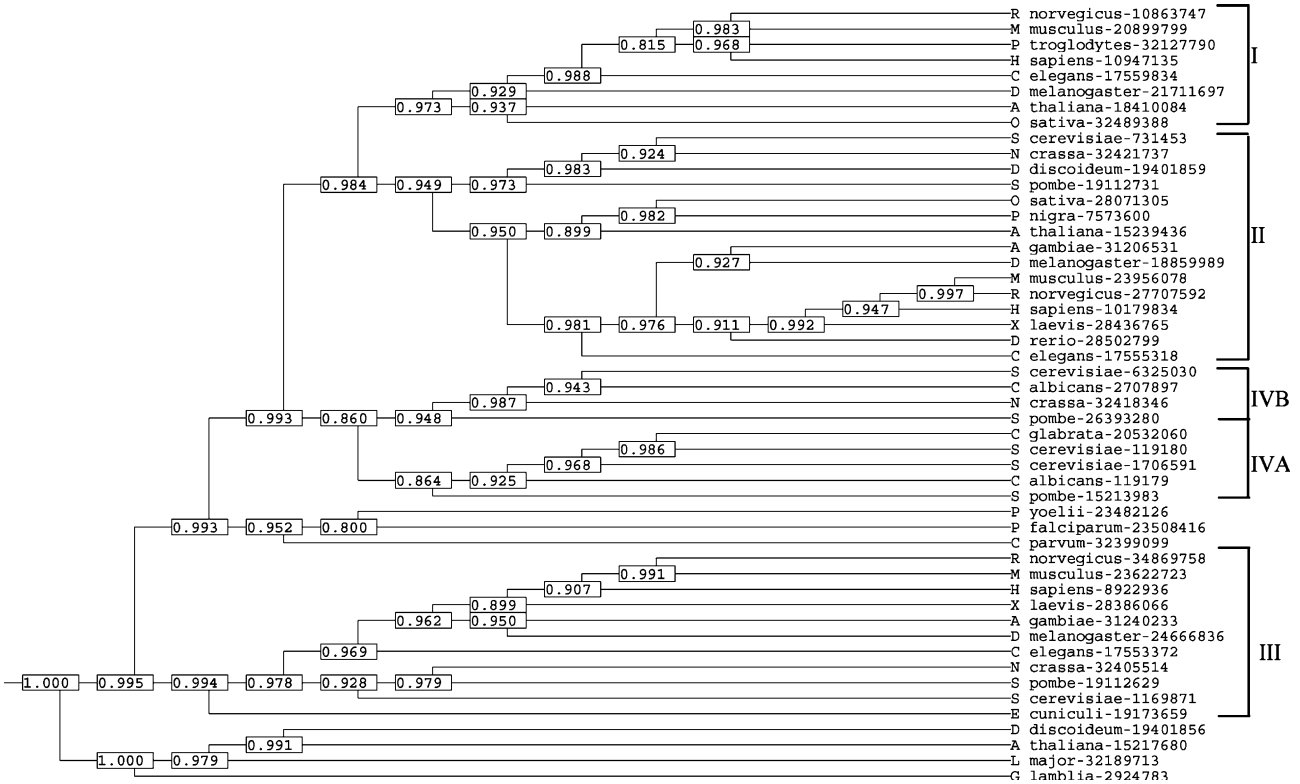

Fig. 1. Phylogenetic tree analysis of eukaryotic ABCF proteins. The tree was prepared as described in the methods employing Genedoc. The four identified classes of ABCF proteins are indicated at the right-hand side. Each sequence is identified by its species and its GenBank accession number. Each branch point is also annotated with the score obtained from 1000 bootstrapping iterations of the alignment. Thus, a score of 1.0 indicates that a branch point was obtained for all 1000 operations.

from the E-site, thus stimulating yeast protein synthesis. The additional cluster of ABCF proteins (Class IVB in Fig. 1) contains yeast proteins believed to be important in mRNA export from the nucleus, although the mechanism of this remains unknown [26].

Is there functional similarity between eukaryotic translation factors and prokaryotic macrolide drug resistance determinants?

Despite the phylogenetic separation of Classes XII and XIII (which reflects the different intervals between the Walker-A and Signature motifs and the amino acid composition of connecting region between the two NBDs) there appears to be functional overlap. Classes XII and XIII include open reading frames associated with macrolide resistance. Among the genes encoding NBD–NBDs associated with antibiotic resistance are tlrC (tylosin resistance [27]) and lmrC (lincomycin [28]) in Class XII and vga (virginiamycin [29]), and msrA (erythromycin [30]) in Class XIII. The macrolactone compounds act as antibiotic agents by binding to the 23S rRNA subunit, inhibiting either the elongation of the nascent protein chain or inducing premature dissociation of the peptidyl-tRNA from the ribosome [31]. Structural investigations demonstrate binding of macrolides to the internal end of the polypeptide exit tunnel, close to the peptidyltransferase centre (P-site) [32], although the access path of the antibiotics to this site is unknown. The phylogenetic analysis presented here (Fig. 3) suggests that the encoded NBD–NBD proteins identified as resistance determinants for macrolides (Classes XII and XIII) are unlikely to be involved in membrane-spanning transport complexes. Indeed, there exist transport proteins of the major facilitative superfamily involved in antibiotic drug export (e.g., LmrA export of lincomycin [28]). Furthermore, there is no conserved operon structure for the Class XII and XIII open reading frames that includes an MSD. As an alternative, this analysis supports the hypothesis that they prevent macrolide binding to the recognition site on the 23S rRNA subunit, either through a direct interaction of the NBD–NBD protein with the 50S ribosomal subunit itself, through a direct interaction with the macrolide [33], or through an activation of a third factor responsible for inhibiting macrolide binding. In parallel with the eukaryotic ABCF proteins the mechanism of the macrolide resistance conferring proteins is proposed to involve tRNA molecules.

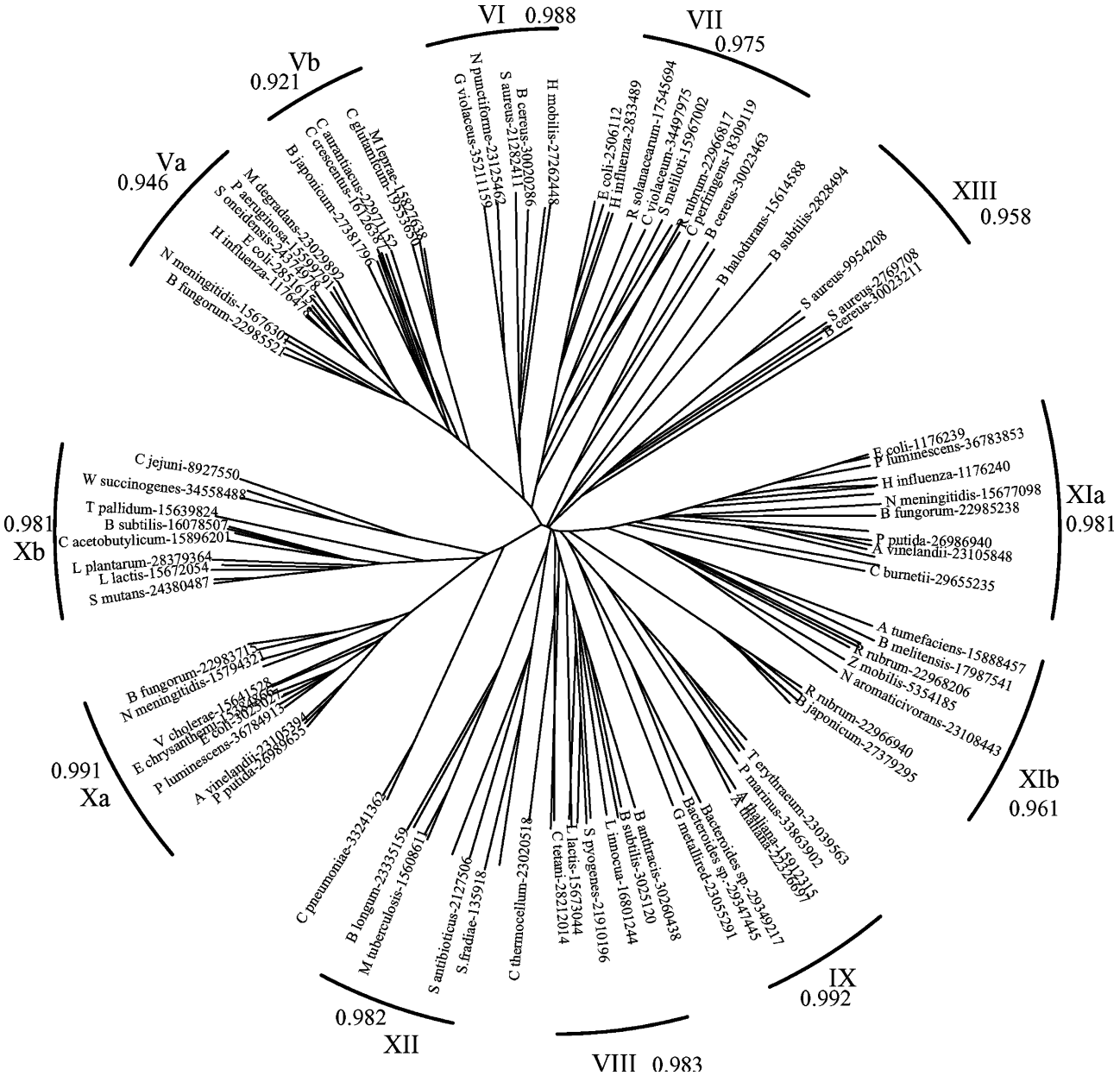

Fig. 2. Phylogenetic analysis of prokaryotic ABCF proteins. The tree was prepared as described in the methods employing the Phylip program to produce the un-rooted tree. The identified classes of ABCF proteins are indicated around the circumference of the tree. For clarity the GenBank sequence identifiers of only a subset of these are shown. Each class is also annotated with the score obtained from 1000 bootstrapping iterations.

Classification of ABCE sequences

Psi-Blast searches of the non-redundant sequence databank with human ABCE [34] as the initial query sequence resulted in the identification of 39 additional homologous sequences. Of the 40 sequences, 22 are from eukarya and the remaining 18 are from archaea (Fig. 4). Within the archaea there was no frequently observed operon structure in which the ABCE gene was located. No eubacterial homologues of ABCE were identified. Among eukaryotes, *A. thaliana* is unique in containing 2 ABCE-like sequences, one each on chromosome 3 and 4. Multiple sequence alignment (Fig. 5) of ABCE sequences enables the identification of several conserved domains within the ABCE sequence. Two of these (residues 75–310 and residues 335–555 in human ABCE) are typical NBDs [3], containing the anticipated sequence motifs (over 90% identify across the 40 ABCE sequences, Fig. 4). N-terminal to the two NBDs is a highly conserved, potential iron–sulphur binding domain (residues 55–65 in human ABCE), which has highest identity with the 4Fe–4S sub-type of ferredoxin

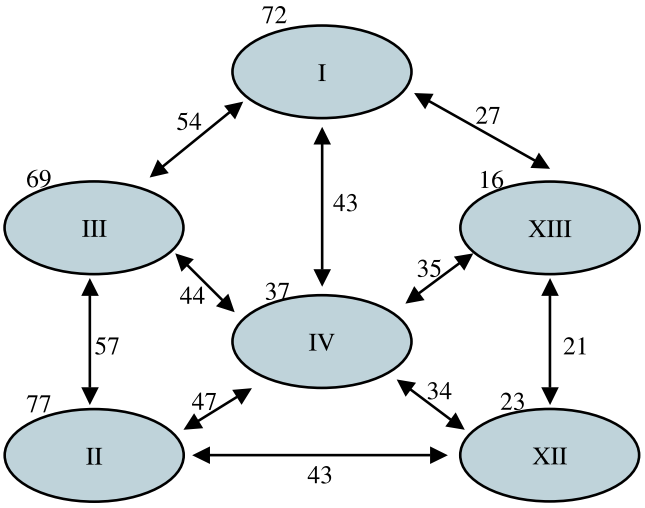

Fig. 3. Representation of the degree of similarity between 6 of the 13 classes of ABCF proteins. The four eukaryotic classes and the macrolide resistance conferring groups are represented by shaded ovals. The number to the top left of each oval is the mean Z score [7] for that class, while numbers adjacent to the connecting arrows are the mean Z score for pairwise comparisons between two classes.

sequence [35]. The CxxCxxCxxxC motif is conserved in 39 ABCE sequences, with *Halobacterium halobium* having an additional two amino acids inserted between the first pair of cysteine residues. At the extreme N-terminus of ABCE is a further region with four highly conserved cysteines. This region (residues 14–30 in human ABCE) is conserved in all 40 ABCE homologues and has the sequence [D/E]xCxPxxCxxxCxxxCP. This spacing of four cysteine residues is not previously described within Fe–S-binding proteins and shares no similarity with any classified motifs on the Pfam database [35]. The presence of two candidate metal-binding domains in ABCE proteins suggests that the activity of the protein may be mediated by redox potential. Two final regions of high sequence identity are a 30–35 amino acid stretch located between NBD1 and NBD2, and the C-terminal tail of similar length. Both of these sequences are unique to members of the ABCE family.

Is ABCE an inhibitor of RNase L?

The annotation of sequence data frequently interprets the ABCE family as inhibitors of RNase L, an interferon-induced ribonuclease potentiated by oligoadenylate and double-stranded RNA. Thus, ABCE has been posited as being an inhibitor of the interferon-mediated response to viral infection [34]. In an attempt to determine whether sequence data could produce hypotheses for the domains in ABCE responsible for interacting with RNase L, database searches were performed to identify candidate RNase L homologues. Psi-Blast searches of the non-redundant sequence database, employing low complexity filtering to mask the ankyrin repeats of RNase L, identified only a human, mouse, and rat sequence. No potential RNase L homologues were identified in any further eukaryotes or in the archaea which possessed a candidate ABCE gene. The identification of RNase L in only 3 of the 40 organisms in which ABCE, a proposed inhibitor of RNase L, is present suggests that this cannot be the only functional

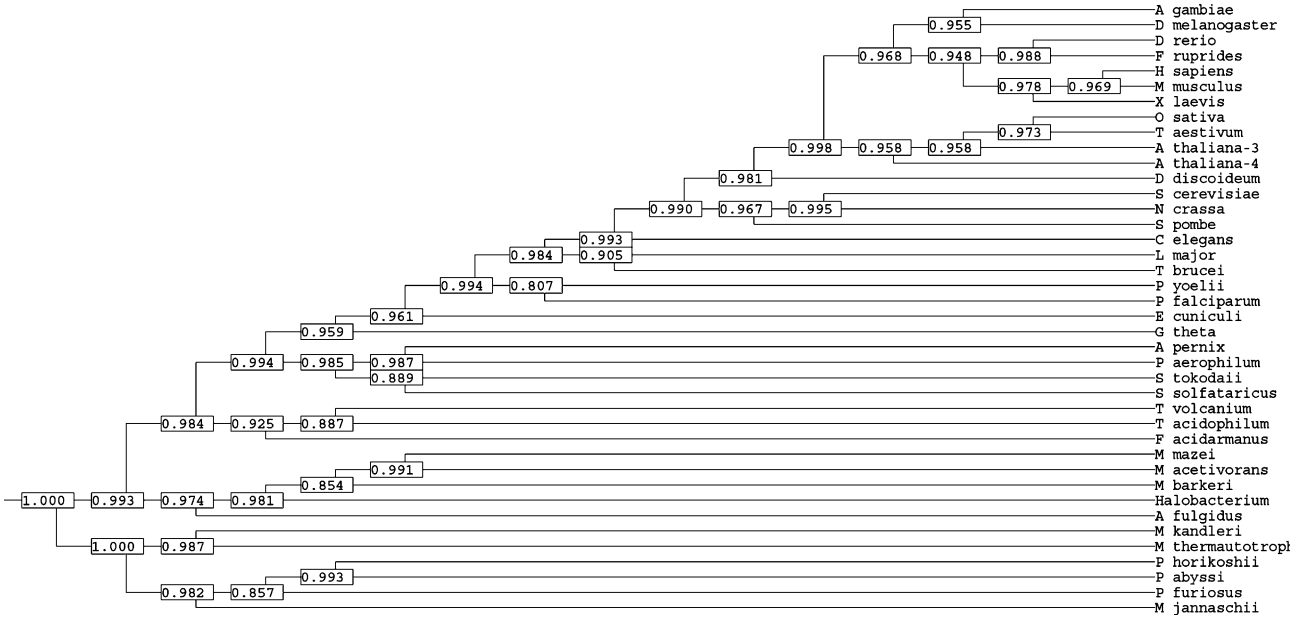

Fig. 4. ABCE phylogeny and sequence motifs. Phylogenetic tree of 40 ABCE sequences identified in the non-redundant sequence database. The numerals '3' and '4' denote the chromosomal location of the two ABCE members from *A. thaliana*.

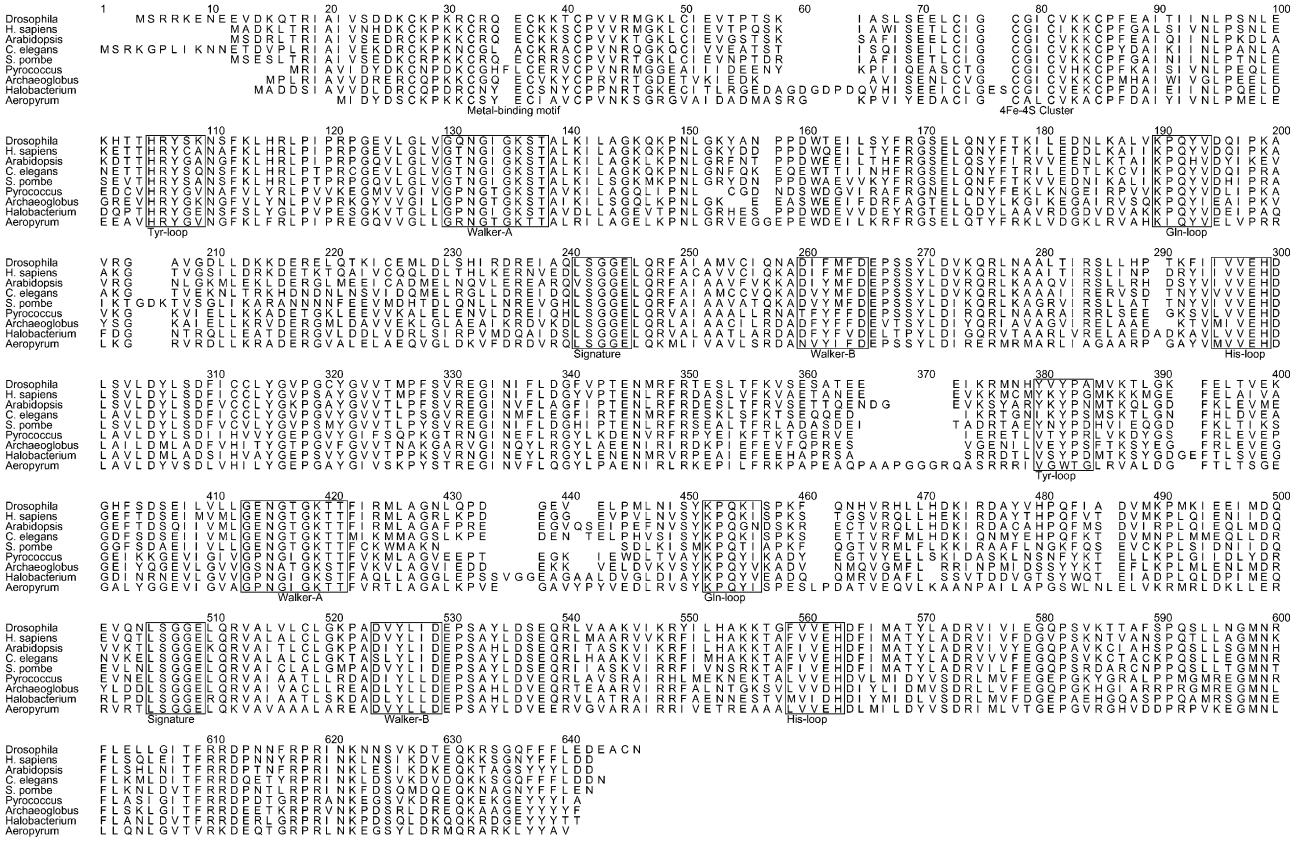

Fig. 5. Multiple alignment analysis of ABCE sequences. Nine ABCE sequences representative of eukarya and archaea were aligned as described in the text and the alignment rendered with the program Alscript [38]. The numbering corresponds to the position in the alignment. Conserved cysteine residues in proposed metal-binding domains are denoted by a shaded background. The positions of the conserved motifs in the two NBDs are identified by boxes.

role of ABCE. More recently, it has been postulated that the ABCE protein may be necessary for the assembly of Gag polypeptides into immature HIV-1 capsids [36]. These two functions could be related. Indeed, down-regulation of the RNase L pathway has been described as a consequence of viral infection and this has been associated with increased levels of ABCE protein [37]. The analysis presented here suggests further research is required to establish the function of ABCE in organisms apparently lacking a RNase L pathway.

Summary

The diversity of proteins containing ABC transporter-like NBDs demonstrates the inherent flexibility of this domain. Advances in the understanding of the role played by NBDs in the transport of substances across cell membranes and in DNA repair have come from biochemical and structural studies. The challenge remains to determine the exact mechanisms of the twin-NBD proteins discussed here, the structural basis of their interaction with ribosomes, and their role in the response to viral infection.

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