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# The phylogenetic distribution of frataxin indicates a role in iron-sulfur cluster protein assembly

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**Much has been learned about the cellular pathology of Friedreich's ataxia, a recessive neurodegenerative disease resulting from insufficient expression of the mitochondrial protein frataxin. However, the biochemical function of frataxin has remained obscure, hampering attempts at therapeutic intervention. To predict functional interactions of frataxin with other proteins we investigated whether its gene specifically co-occurs with any other genes in sequenced genomes. In 56 available genomes we identified two genes with identical phylogenetic distributions to the frataxin/*cyaY* gene: *hscA* and *hscB*/*JAC1*. These genes have not only emerged in the same evolutionary lineage as the frataxin gene, they have also been lost at least twice with it, and they have been horizontally transferred with it in the evolution of the mitochondria. The proteins encoded by *hscA* and *hscB*, the chaperone HSP66 and the co-chaperone HSP20, have been shown to be required for the synthesis of 2Fe-2S clusters on ferredoxin in proteobacteria. *JAC1*, an ortholog of *hscB*, and *SSQ1*, a paralog of *hscA*, have been shown to be required for iron-sulfur cluster assembly in mitochondria of *Saccharomyces cerevisiae*. Combining data on the co-occurrence of genes in genomes with experimental and predicted cellular localization data of their proteins supports the hypothesis that frataxin is directly involved in iron-sulfur cluster protein assembly. They indicate that frataxin is specifically involved in the same sub-process as HSP20/Jac1p.**

## INTRODUCTION

The sequencing of complete genomes has provided the opportunity not only to interpret the function of a protein within its proteomic context, but also to predict new functional interactions between proteins using comparative genome analysis (1). Specifically it has been proposed (2) and demonstrated (3) that proteins of genes that co-occur with each other in genomes (they are either both present or both absent) tend to functionally interact. The co-occurrence of genes in genomes (also called 'phylogenetic profiles') can be used as a tool to predict functional interactions between their proteins (4,5). Such functional interactions span a wide variation of interactions, including direct physical interactions between the proteins, but also less direct ones, such as being part of the same metabolic pathway or biological process (5). When there is prior knowledge about a protein's involvement in a process, yet the exact function of the protein is not known, the co-occurrence of genes can more specifically pinpoint in which sub-process the protein plays a role (6,7). Here we use genome comparisons to predict functional interactions for frataxin, a mitochondrial protein that has no detectable homologs with known function and that presently has a unique fold (8,9). Severely reduced levels of frataxin cause the disease Friedreich's ataxia (10), which is characterized by degeneration of large sensory neurons and spinocerebellar tracts, cardiomyopathy and increased likelihood of diabetes (11). In mitochondria, reduced levels of frataxin result in the absence of iron-sulfur cluster (isc) dependent enzymes, accumulation of iron deposits, DNA damage and oxidative stress (12). Based on such observations the main hypothesis about frataxin's function is that it is directly involved in iron homeostasis of the mitochondria. Alternatively it has been proposed that frataxin is involved in isc assembly on iron-sulfur proteins (13). Recent findings that the yeast ortholog of frataxin precipitates with iron support the first hypothesis (14); however, they could not be reproduced with purified human frataxin itself (8). Here we show that the frataxin gene and its orthologs (*cyaY* in bacteria) have the same phylogenetic distribution as the chaperones *hscA* and *hscB/JAC1*, supporting a direct role in the assembly of isc proteins, rather than in iron homeostasis.

## RESULTS

### Co-occurrence of frataxin with proteins involved in isc assembly in the bacteria

Orthologs of the human frataxin gene are found in all sequenced eukaryotic genomes, and in most proteobacteria (purple bacteria), specifically in all but one of the sequenced γ-proteobacteria, in all of the sequenced β-proteobacteria and in one of the sequenced α-proteobacteria: *Rickettsia prowazekii*, the closest fully sequenced relative to the ancestor of the mitochondria (Fig. 1). That frataxin is only

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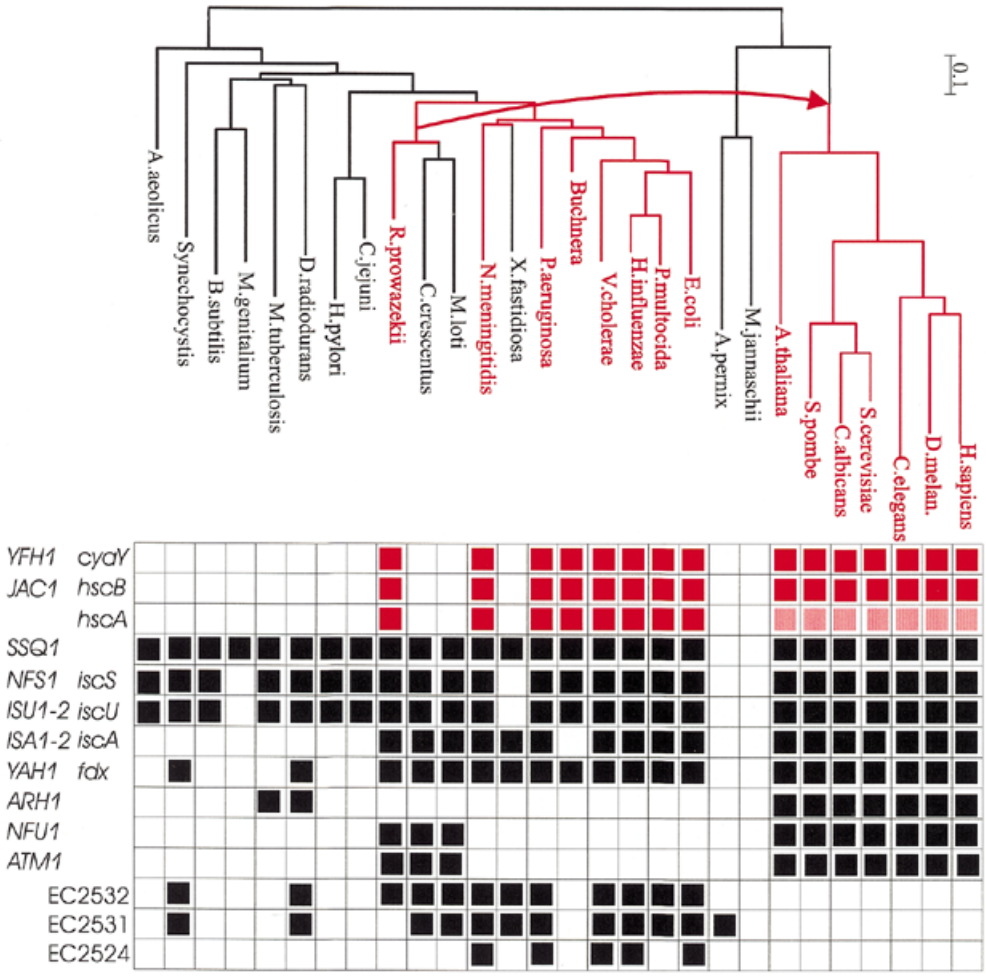


**Figure 1.** A history of isc assembly. The phylogenetic distribution of genes involved in isc assembly in proteobacteria and eukaryotes is summarized. Several other prokaryotic species, including the archaea *Methanococcus jannaschii* and *Aeropyrum pernix*, have been included for reference. The first two columns give the gene names in *S.cerevisiae* and prokaryotes. The genes for frataxin (*YFH1* in *S.cerevisiae*, *cyaY* in bacteria), *hscA* and *hscB* have identical distributions and are indicated in red. Proteins encoded by orthologs of *hscA* in the eukaryotes are not located in the mitochondria and are therefore indicated in pink. Black squares indicate the presence in the various genomes of full-length orthologs of the other genes implicated in isc assembly. The phylogeny was constructed using complete genome comparisons (47), and is consistent with standard 16S ribosomal RNA phylogenies. Orthology relations were determined by selecting all the homologs of a protein using iterative PSI-BLAST searches (44), and subsequent manual inspection of phylogenetic trees constructed with clustalX (45). Short genes that were conspicuously missing from a genome were searched for at the DNA level, using TBLASTN (44). This procedure revealed, for example, the presence of an ortholog of *hscB* in *Neisseria meningitidis* MC58 that was not yet annotated as a gene. The taxa in the phylogeny that contain the frataxin/*cyaY* gene, *hscA* and *hscB*, are in red, and the horizontal gene transfer that accompanied the origin of the mitochondria is indicated with a red arrow. Frataxin/*cyaY*, *hscA* and *hscB* appear to have been lost from *X.fastidiosa* and from the lineage leading to *M.loti* and *C.crescentus*. An alternative for the latter is that *Rickettsia prowazekii* has gained them by horizontal transfer. The phylogenetic trees of the individual sequences (e.g. Fig. 2) are however more consistent with gene-loss in *M.loti* and *C.crescentus* than with horizontal transfer. Species names in full: *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae, Candida albicans*, *Schizosaccharomyces pombe*, *Rickettsia prowazekii, Caulobacter crescentus*, *Melorhizobium loti*, *Neisseria meningitidis*, *Xylella fastidiosa*, *Pseudomonas aeruginosa, Haemophilus influenzae*, *Pasteurella multocida*, *Vibrio cholerae, Escherichia coli, Campylobacter jejuni*, *Helicobacter pylori*, *Deinococcus radiodurans*, *Mycobacterium tuberculosis*, *Mycoplasma genitalium*, *Bacillus subtilis, Aquifex aeolicus, Methanococcus jannaschii*, *Aeropyrum pernix*.

present in the proteobacterial clade led to the proposal that in eukaryotes the protein is targeted to the mitochondrion (15), which was substantiated by subsequent experimental evidence (10,16–18).

To find possible interaction partners for frataxin we determined the phylogenetic distribution of all genes of the smallest genome that contains the frataxin gene: the intracellular symbiont proteobacterium *Buchnera* (Materials and Methods). As summarized in Figure 1, only two genes have a phylogenetic distribution that is identical to that of frataxin: the chaperone pair *hscA* and *hscB/JAC1* (Fig. 1). In bacteria this pair is part of the so-called isc assembly operon (19) that in *Escherichia coli* encodes nine proteins: a hypothetical RNA methylase (*E.coli* gene no. EC2532), a hypothetical helix–turn–helix containing transcriptional regulatory protein (EC2531), IscS, IscU, IscA, HscB, HscA, fdx (a 2Fe-2S ferredoxin), and a third hypothetical protein (EC2524). A number of the encoded proteins appear to be involved in the generation of iscs on ferredoxin in *E.coli*: IscS, IscA, HscA, HscB and EC2524 (20). The clustering of these genes into one operon

(actually two operons in *Rickettsia* and *Neisseria*) has, within the bacteria, the same phylogenetic distribution as the frataxin gene. However, at the level of the individual genes this only applies to *hscB* and *hscA*. Other genes from the cluster are either more widespread in the bacteria (EC2532, EC2531, *iscS*, *iscU*, *iscA* and *fdx*) or less widespread (EC2524).

### Evolution of the eukaryotic isc assembly in mitochondria

Eukaryotic orthologs, and in one case a paralog, of most of the bacterial isc proteins have been implicated in isc generation in yeast. These are: Nfs1p (21) (ortholog of IscS), Isu1p and Isu2p (22) (orthologs of IscU), Isa1p and Isa2p (23,24) (orthologs of IscA), Yah1p (25) (ortholog of the fdx gene product), Ssq1p (26) (paralog of HscA; see below and Fig. 2) and Jac1p (26,27) (ortholog of HscB). EC2532, EC2531 and EC2524 have no orthologs in the eukaryotes and appear not to have been part of the massive horizontal gene transfer that accompanied the origin of the mitochondria. Besides the frataxin ortholog Yfh1p, several other yeast proteins without orthologs in the bacterial *isc* operon have also been implicated in isc assembly. Nfu1p (22) and Atm1p (28) (an ABC transporter that exports the iscs from the mitochondria) have full-length orthologs in all the sequenced α-proteobacteria, while the adrenodoxin/ferredoxin reductase Arh1p (29) has orthologs in some gram-positive bacteria. With a couple of exceptions the isc assembly proteins in mitochondria have thus been derived from the proteobacterial isc operon, implying a considerable conservation of the isc assembly from the proteobacteria to the mitochondria (22,30). Within the sequenced eukaryotes the isc set of proteins appears perfectly conserved; the yeast proteins implicated in isc assembly have orthologs in all the other sequenced eukaryotic genomes (Fig. 1), although some variation might exist in the compartmentalization of the isc assembly (31).

### A paralogous displacement in isc assembly in eukaryotes

Interestingly Ssq1p, the HSP70 protein that functions in the yeast mitochondrion in isc assembly, is not orthologous to HscA, but rather paralogous to it (Fig. 2). Orthologs of *hscA* are present in all sequenced eukaryotic genomes but their proteins have not been observed in mitochondria. In addition, the protein localization prediction program Psort (32) predicts the proteins of this orthologous group to be cytoplasmic. This switching of DnaK with HscA in the evolution of the eukaryotic cell suggests that the functioning of DnaK/HscA in isc assembly is not very substrate specific. It should be noted here that the substrate specificity of the DnaK-DnaJ pair is largely determined by DnaJ (33). Furthermore, in the evolution of HscA-HscB from DnaK-DnaJ, it is HscB that has undergone the largest change, having only retained the N-terminal, DnaK interacting domain from DnaJ, while the middle and C-terminal domains have been replaced by a heterologous three helix bundle domain (34). In contrast, HscA is a full length homolog of DnaK. It has retained functionality as a chaperone for standard substrates as such as rhodanese or citrate synthase (35). Relative to HscB, HscA has thus retained more of the structure and function of its ancestral protein. HscA might thus have been easier to replace by its ancestor DnaK than HscB by its ancestor DnaJ in the evolution of isc assembly. Note also that while in the fungi and in *Arabidopsis* there have been a number of gene duplications of the mitochondrial HSP70 proteins, leading for example to the three HSP70 proteins of this orthologous group in *Saccharomyces cerevisiae*—among which the functionally differentiated Ssq1p and Ssc1p, in the metazoa no such duplications can be detected. *Homo sapiens* has only one member of this orthologous group of proteins.

### Co-evolution of the HscA, HscB and frataxin genes

Given their widespread phylogenetic distribution, EC2532, EC2531, *iscS*, *iscU* and *fdx* are likely to have existed in the bacteria before the *dnaK-dnaJ* duplication gave rise to *hscA-hscB*, apparently in the proteobacterial clade. At about the same time that frataxin was invented, this chaperone pair has thus been added to a preexisting set of isc proteins that are likely to have already functioned in isc assembly. Subsequently the *hscA-hscB* gene pair and frataxin have been lost in *Xylella fastidiosa* and in the lineage leading to *Melorhizobium loti* and *Caulobacter crescentus* (Fig. 1). Such co-loss of genes increases the likelihood that the proteins they encode are functionally linked (6,7). Furthermore, a large fraction of the set of isc genes have been transferred together with the frataxin gene to the nuclear genome of eukaryotes after the symbiosis of an α-proteobacterium with the predecessor of the eukaryotes that led to the mitochondria. Note that one other gene has been invented at about the same time as *hscA*, *hscB* and frataxin/*cyaY*: *iscA* (through a gene duplication within the HesB family). However, its subsequent evolutionary history is different. For example, *IscA* has been lost from Buchnera while *hscA*, *hscB* and frataxin/*cyaY* have been retained in this species.

### Molecular functions of the likely interaction partners of frataxin: HscA/Ssq1p and HscB/Jac1p

Based both on experimental evidence and on their phylogenetic distribution, the isc proteins can be divided into subsets. On the one hand IscS/Nfs1p, IscU/Isu1-2p and IscA/Isa1-2p function in the assembly of iron-clusters themselves. IscS/Nfs1 are cysteine desulfurases (21). IscU/Isu1-2p serve as scaffolds for isc biosynthesis (36). IscA has been shown to interact with the holoform of ferredoxin (37), and conserved, essential cysteines in Isa1p hint at a role in iron-binding (23). On the other hand, although HscA/Ssq1p and HscB/Jac1p have consistently been shown to be involved in the generation of iscs in bacteria and mitochondria (20,26,27), their exact molecular functions have not been elucidated. The homology of HscA/Ssq1p and HscB/Jac1p with the protein pair DnaK and DnaJ suggests that they function as chaperones, possibly facilitating the folding of iron-sulfur proteins. One substrate of the chaperone pair is IscU. Physical interaction between IscU and HscA/HscB has been observed in *E.coli* (38). HscA/HscB could serve as specialized chaperones for IscU (35), but might also facilitate the transfer of Fe/S clusters formed in IscU to apoacceptor proteins (35). While HscA is a full-length homolog of DnaK, HscB shares only the N-terminal, DnaK-binding domain with the classical DnaJ. In HscB, the zinc-finger middle domain and the C-terminal domain that in DnaJ are involved in substrate binding (39,40) have been replaced

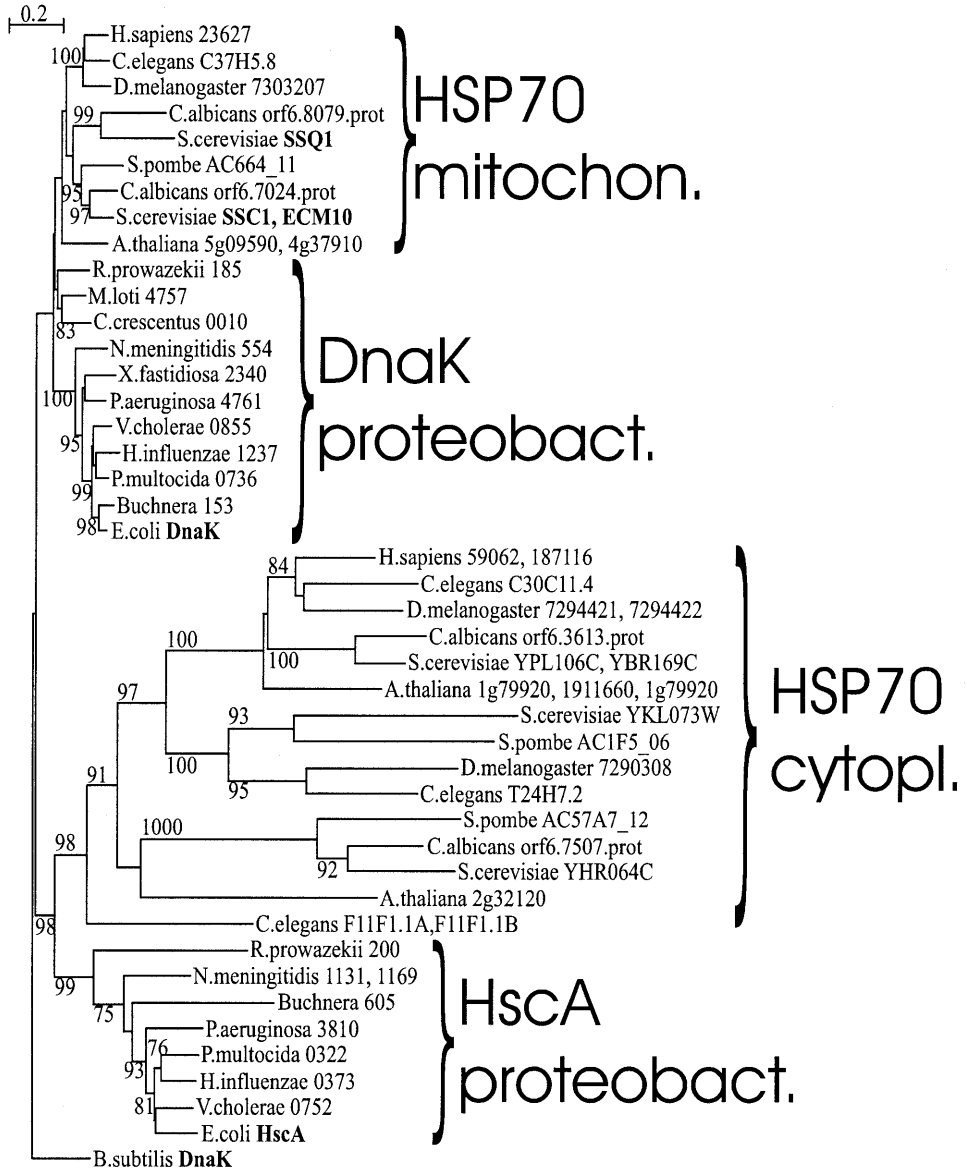


**Figure 2.** A paralogous displacement in HSP70 proteins involved in isc cluster formation in eukaryotes relative to bacteria. The figure depicts the phylogenetic relations between the HSP70 proteins that have been implicated in isc assembly in proteobacteria (HscA proteobact.), their orthologs in eukaryotes (HSP70 cytopl.), HSP70 proteins implicated in isc genesis in mitochondria (HSP70 mitochon.), their orthologs in proteobacteria (DnaK proteobact.), and an outgroup, *B.subtilis* DnaK. With each gene is given the number it has in the genome. The phylogeny indicates that the yeast protein Ssq1p is not orthologous to HscA, but has rather descended directly from the bacterial DnaK. Still, all the eukaryotic genomes contain an ortholog of HscA. No experimental evidence about these proteins is available, alhough their origin suggests a possible role in isc protein assembly in the cytoplasm. Note that in contrast to the fungi and *Arabidopsis*, no gene duplications of the mitochondrial HscA orthologs can be detected in metazoan genomes. The tree was generated with ClustalX (45), selecting only positions that were present in all sequences, and correcting for multiple substitutions. Bootstrap values larger than 75/100 are shown. They are based on 1000 bootstraps that were subsequently rounded off. In the case of species-specific gene duplications that were found with bootstrap values larger than 90/100, the gene names were merged into one branch (e.g. *S.cerevisiae* SSC1, ECM10).

with a three helix bundle coiled-coil like structure (34), of which the function is not yet clear.

## DISCUSSION

The identical phylogenetic distribution of the frataxin gene with *hscA/SSQ1* and *hscB/JAC1* suggests that frataxin plays a role in the same stage of the process of isc protein assembly as the HscA-HscB chaperone system, possibly as co-chaperone, or in protecting the sulfhydryl groups of iron-sulfur apoproteins. One possibility is that frataxin plays a role in the selection of the substrate. It could replace the substrate-selecting function of the middle and C-terminal domains of DnaJ that are missing in HscB/Jac1p. The conservation of a string of negatively charged residues on the surface of the protein (8) supports the hypothesis of a role in peptide binding. Alternatively it could

interact with HscA/Ssq1p. However, the paralogous displacement of HscA with Ssq1p in the evolution of isc assembly makes this interaction less likely to be specific. Nevertheless, Ssq1p has been shown to be involved, together with Ssc1p, in the maturation of the yeast frataxin ortholog, Yfh1p (41). It should be noted that the types of functional interaction that correlate with the co-occurrence of genes in genomes include a large variation of functional interactions, and do not necessarily reflect physical interaction, but rather that the proteins are involved in the same (sub)process.

In any case, the strict co-occurrence of the frataxin gene with *hscA/SSQ1* and *hscB/JAC1* strongly supports a direct role of frataxin in isc protein assembly. The hypothesis that frataxin is directly involved in isc assembly and that the accumulation of iron in frataxin-deficient cells is only a secondary effect is not new (13). Recent evidence from yeast supports a direct role of frataxin in isc assembly (26), while in frataxin-deficient mouse cells the accumulation of iron is secondary to deficiency of isc proteins (42). Based on the co-occurrence of genes in genomes and their encoded proteins having the same distribution in the eukaryotic cell, we can, however, be more specific in our predictions: frataxin should function in conjunction with HscB/Jac1p.

## MATERIALS AND METHODS

The determination of orthology relations between the genes in *Buchnera* and the other 55 sequenced genomes was done in two steps. First we compared all the predicted protein sequences from *Buchnera* with those from the other genomes using the Smith–Waterman (43) algorithm and selected bidirectional best, not-overlapping (to include the possibility of fission/fusion of genes), homologs ($E < 0.01$) (1). This procedure gives a first-order approximation of the orthology relations. Subsequently the 20 genes that had the most similar phylogenetic distribution to that of the frataxin gene were selected for more careful, manual analysis. For those genes we performed iterative PSI-Blast searches (five iterations, $E < 0.001$) (44) to select all family members of these genes. These were aligned using clustalX (45) and Neighbor-joining trees were constructed (46). In the second step we made high quality orthology predictions by manual inspection of these phylogenetic trees. Subsequently groups of orthologous genes were selected that had the most similar distribution to the frataxin gene. This procedure revealed only two genes with the same distribution as frataxin, *hscA* and *hscB/JAC1*; other genes from *Buchnera* have a discrepancy of at least seven in their phylogenetic distribution, i.e. there are seven genomes where either they are present and frataxin is not, or vice versa.

All sequenced prokaryotic genomes (for an overview see www.tigr.org/tdb/mdb/mdbcomplete.html) and those of *S.cerevisiae*, *Drosophila melanogaster* and *Arabidopsis thaliana* were obtained from GenBank (ftp.ncbi.nlm.nih.gov/pub/genomes). The *Schizosaccharomyces pombe* genome was obtained from the European *Schizosaccharomyces* genome sequencing project (http://www.sanger.ac.uk/Projects/S_pombe), *Candida albicans* from the Stanford Genome Technology Center (http://www-sequence.stanford.edu/group/candida/search.html) and the *H.sapiens* genome from Ensembl (http://www.ensembl.org).

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