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ORIGINAL ARTICLE

An evidence of laccases in archaea

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Abstract Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are a diverse group of multicopper oxidases that catalyze the oxidation of a variety of aromatic compounds. Here we present evidence for distribution of laccases among archaea and their probable functions. Putative laccase genes have been found in different archaeal groups that might have branched off early during evolution, e.g. *Haloarcula marismortui* ATCC 43049, *Natronomonas pharaonis* DSM2160, *Pyrobaculum aerophilum* IM2, *Candidatus Nitrosopumilus maritimus* SCM1, *Halorubrum lacusprofundi* ATCC 49239. Most of the archaeal multicopper oxidases reported here are of Type 1 and Type 2 whereas type 3 copper-binding domain could be found in *Pyrobaculum aerophilum* IM2 and *Halorubrum lacusprofundi* ATCC49239. An analysis of the genome sequence database revealed the presence of novel types of two-domain laccases in archaea.

Keywords Archaea · Multicopper oxidase · Laccase · Genome · Cluster of orthologus groups

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Introduction

Laccases are one of the best-known members of the multicopper protein family, also known as benzenediol:oxygen oxidoreductase, EC 1.10.3.2 [1]. They are the model enzymes of multicopper oxidases (MCOs) which participate in (1) cross-linking of monomers, (2) degradation of polymers, and (3) ring cleavage of aromatic compounds [2, 3, 4, 5]. Being the simplest enzyme that combines all three known organic Cu(II) magnetic types in a single molecule, laccase has been particularly well studied with respect to its intramolecular electron transfer reactions [6]. Phylogenetically, laccases are member of MCOs family including ascorbate oxidase (EC 1.10.3.3), cytochrome c oxidase (EC 1.9.3.1), and ceruloplasmin (sometimes referred to as ferroxidase; EC 1.16.3.1). Commonly, a three-domain multicopper laccases have been reported from eukaryotes, e.g., fungi, lichens, plants and insects [4, 7–9]. There are some evidences, however, for its existence in prokaryotes; a protein with typical features of multicopper oxidase enzyme family, which are mainly involved in cell pigmentation and metal oxidation [10, 11]. The first bacterial laccase was detected in the plant root-associated bacterium, *Azospirillum lipoferum*, where it was shown to be involved in melanin formation [12]. The most well-characterized bacterial laccase was isolated from *Sinorhizobium meliloti*, which has been described as a 45-kDa periplasmic protein with isoelectric point at pH 6.2 and ability to oxidize syringaldazine [13].

The completely sequenced archaeal genomes potentially encode many functionally uncharacterized genes for novel enzymes of biotechnological interest [14]. Current decade has witnessed the determination of the complete archaeal

Table 1 Putative archaeal laccase compared with other novel laccases (http://img.jgi.doe.gov)

Genome	Gene	Locus tag	Product	DNA coordinates	GC	Accession	AA	Trans-membrane helices	Signal peptide	IMG term/families
Haloarcula marismortui ATCC 43049	*nirK*	rrnAC2853	Nitrite reductase copper-containing protein	2531326..2532411 (-) (1086bp)	0.65	AAV47603	361	No	Yes	Copper-containing nitrite reductase Cupredoxin Blue copper subtype Multicopper oxidase types
	Pan 1a	rrnAC1378	Membrane protein Pan 1	1227027..1228178 (-) (1152bp)	0.63	AAV46307	383	No	No	Copper ion binding Electron carrier activity Oxidoreductase activity Twin-arginine translocation pathway signal; Cupredoxin Blue (type 1) copper subtype
Natronomonas pharaonis DSM2160	*nirK*	NP1598A	Nitrite reductase copper containing	773076..774164 (+) (1089bp)	0.65	YP_326457	362	No	No	Nitrite reductase, copper-containing
Pyrobaculum aerophilum IM2	*PAE1888*	PAE1888	Multicopper oxidase	1114128..1115561	0.50	AAL63794	477	No	No	Copper ion binding Oxidoreductases activity Cupredoxin Multicopper oxidase, copper-binding site Multicopper oxidase, type 2 Multicopper oxidase, type 3 (preliminary) Laccase
Candidatus Nitrosopumilus maritimus SCM1	-	NmarDRAFT_0728	Multicopper oxidase, Type 3	295350..2964405 (-) (1056 bp)	0.38	ZP_02024104	351	Yes	Yes	Copper-containing nitrite reductase, Multicopper oxidase, type 2 Multicopper oxidase, type 3
Halorubrum lacusprofundi ATCC 49239	-	HlacDRAFT_1345	Multicopper oxidase, Type 3	771769..773064 (+) (1296 bp)	0.65	ZP_02015243	431	No	Yes	Blue (Type 1) copper subtype Multicopper oxidase, type 2 Multicopper oxidase, type 3

Table 1 (Continued)

Genome	Gene	Locus tag	Product	DNA coordinates	GC	Accession	AA	Trans-membrane helices	Signal peptide	IMG term/families
Yersinia enterocolitica enterocolitica 8081	*yacK*	YE0712	Hypothetical Protein	827646..829247 (+) (1602bp)	0.53	YP_ 001005057	533	No	No	Multicopper oxidase, copper-binding site Twin-arginine translocation pathway signal Cupredoxin Multicopper oxidase, type 2 Multicopper oxidase, type 3
Pichia stipitis CBS 6054	*FET3.1*	PICST_89638	Multicopper oxidase	131793..136959 (-) (5167 bp)	0.42	XP_ 001385046	626	Yes	Yes	Cupredoxin Multicopper oxidase, type 2 Multicopper oxidase, type 3
Escherichia coli APEC O1	*pco A*	APECO1_ OIR119.2	Copper resistant protein PcoA	135300..137123	0.52	YP_ 001481473	607	No	Yes	Twin-arginine translocation pathway signal Copper resistant protein-CopA family
Burkholderia pseudomallei 1710 b	*aniA*	BURPS1710b_ A0477	Multicopper oxidase domain protein	659851..661314	0.68	YP_335636	487			Copper ion binding Electron carrier activity Heme binding Iron ion binding Nitrite reductase Oxidoreductases activity
Burkholderia mallei 10399		Bmal 10_ 03000556	Hypothetical protein	176678..178141	0.68	ZP_01346491	487			
Bacillus pumilus	*cotA*	BPUM_0542	Outer spore coat protein A	577315..578844	0.44	YP_ 001485796	509			
Saccharomyces cerevisiae	*FET5*	YFL041 W	Multicopper oxidase	49139..51007 (+) (1869bp)	0.42	NP_116612	622	No	Yes	Copper ion binding Ferroxidase activity Iron ion binding Metal ion binding Oxidoreductase activity Protein binding

Table 1 (Continued)

Genome	Gene	Locus tag	Product	DNA Coordinates	GC	Accession	AA	Trans-membrane helices	Signal peptide	IMG term/families
Neruospora crassa OR74A	*NCU04528.1*	NCU04528.1	Laccase precursor	78434..80350 (-) (1917bp)	0.59	XP_956939	619	No	Yes	
Cryptococcus neoformans var. *neoformans* JEC 21	-	CNM02420	Acidic Laccase	727710..730327 (-) (2618bp)	0.49	XP_568259	640	Yes	Yes	Multicopper oxidase, type 1 Multicopper oxidase, copper-binding site Cupredoxin Multicopper oxidase, type 2 Multicopper oxidase, type 3 (Preliminary) Laccase
Drosophila melanogaster	*CG32838*	Dmel_CG32838	CG32838	1341942..1342517 (+) (576 bp)	0.40	NP_724414	174	No	No	Cupredoxin Multicopper oxidase, type 2
Homo sapiens	*HEPH*	RP13-238N7.1	Hephaestin	65730180..65834740 (+) (4279 bp)	0.39	NP_620074	1158	No	No	Iron ion binding, metal ion binding, oxidoreductases activity
Ratus norvegicus GK/Ox	*Cp*	Cp	Ceruloplasmin	97809765..97854827 (+) (3700 bp)	0.37	NP_036664	1059	No	Yes	Multicopper oxidase, type 1 Multicopper oxidase, type 2 Multicopper oxidase, type 3 Cupredoxin, copper-binding site

genome. A number of other genome projects are underway and it is no exaggeration to consider it the origin of a new science - a genome-based biology. The availability of complete archaeal genome and huge industrial applications of natural and modified archaeal enzymes has resulted in the exploration of biocatalysts from diverse sources. Surprisingly, till date, there are no reports of archaeal laccase gene.

Materials and methods

To identify putative archaeal laccases, we applied in silico data mining, followed by exhaustive BLAST search of non-redundant (nr) protein sequence database (http://www.ncbi.nlm.nih.gov/) and completed whole genome sequence provided by the Department of Energy- Joint Genome Institute (JGI) (http://img.jgi.doe.gov). The database of Cluster of Orthologus Groups of proteins (COGs) was used as a tool for phylogenetic classification of the proteins encoded in complete genomes of bacteria, archaea, and eukaryotes (http://img.jgi.doe.gov. and http://www.ncbi.nlm.nih.gov/cog) [15].

Signal peptide sequence was predicted using SignalP (http://www.cbs.dtu.dk/services/signalp) and hydrophobicity analysis using the dense alignment surface algorithm (http://www.biokemi.su.se/~server/DAS/). Enzyme class (putative laccases) obtained from cluster of orthologous groups was predicted using ProtFun (http://www.cbs.dtu.dk/servises/ArchaeaFun/).

Three reconstruct methods, edit sequence, megalign, and tree view, were used to find the evolutionary trees or trees that best account for the observed variation in the group of protein sequences (DNASTAR, Inc., 3801 Reagent Street, Madison, WI 53705 USA). Each of these methods uses a different type of analysis and the reliability of the prediction was evaluated by the random re-sampling of the alignment (nucleotide substitution value).

Results and discussion

Collection of 18 COGs from archaea, bacteria, and eukaryotes was compiled (http://img.jgi.doe.gov. and http:// www.ncbi.nlm.nih.gov/cog) (Table 1). The COGs were consistency of genome-specific best hit to the results of an exhaustive comparison of all protein sequences from the genomes. Genome-specific best hit resulted in very exhaustive genomic information of diverse multicopper oxidases. We also built trees grouping organisms based on the overall occurrence of molecular features, i.e. COG throughout the genomes of different archaea, bacteria, fungi (ascomycetous and basidomycetous), different forms of yeast, insects and mammals. Broadly these characteristics could be orthologs, homologs or folds. We focused on orthologs, i.e. multicopper oxidase from diverse archaeal, bacterial and eucaryal sources.

Since our interest lies in the archaeal laccases (multicopper oxidase), we included fungal and bacterial COGs for comparative characterization and evolutionary distances among well-established laccases with the putative laccases from archaea. Mammalian multicopper oxidase, a homolog, was observed as outgroup in the rooted phylogenetic tree (Fig. 1). Although, they form different gene products i.e. ceruloplasmin in *Rattus norvegicus* and hephaestin in *Homo sapiens* but they share a common clade (Table 1). Laccase (cotA) from *Bacillus subtilis*-168 and *Bacillus pumilus*-SAFR-032 were found to share a common clade and close ancestry with multicopper oxidase from *Pyrobaculum aerophilum*, an archaea. Moreover, *P. aerophilum* was also found to be evolutionary related to *Escherichia coli* APEC O1 (laccase) and *Yersinia pestis* KIM (hypothetical protein). Well-known laccases from *Trametes versicolor* were found to be closely related to *Neurospora crassa* OR74A, *Cryptococcus neoformans* var. *neoformans* JEC21 and *Drosoplila melanogaster*, a common fruit fly. Multicopper oxidase from different yeast, i.e. FET3_Yeast, *Pichia stipitis* CBS6054 (FET3.1), and *Saccharomyces cerevisiae* (FET5) share a common phylogenetic position. An unusual evolutionary history was also established between pathogenic proteobacteria, i.e. *Burkholderia mallei* and *Burkholderia pseudomallei* and an archaeal species, i.e. *Haloarcula marismortui* ATCC 43049 and *Natronomonas pharanis* DSM2160 (Fig. 1).

A hydrophobicity analysis using the dense alignment surface algorithm suggested that putative archaeal laccases lack transmembrane regions, which verify that they are soluble protein (Table 1). The putative archaeal laccases identified here include NirK (nitrite reductase) from *H. marismortui* and *N. pharaonis*; Pan1a (membrane protein) from *H. marismortui*; PAE1888 (multicopper oxidase) from *P. aerophilum* (Table 1). Similarities between these proteins and multicopper oxidases were recognized, however, the proteins have not been analysed for laccase activity. Also, we have found putative laccases in representatives of bacteria, fungus, blue-green algae, insect and mammals.

Based on the protein sequence analysis and E value cut-off of 10^{-7}, fixed earlier by Alexandre and Zulin [10]. We could find that most of the BLAST search represented laccases with E value within the cut-off limit (Table 2). Archaeal multicopper oxidase (putative laccase) sequence annotation using Pfam protein families database (http://pfam.sanger.ac.uk/) resulted in high Bits score, which

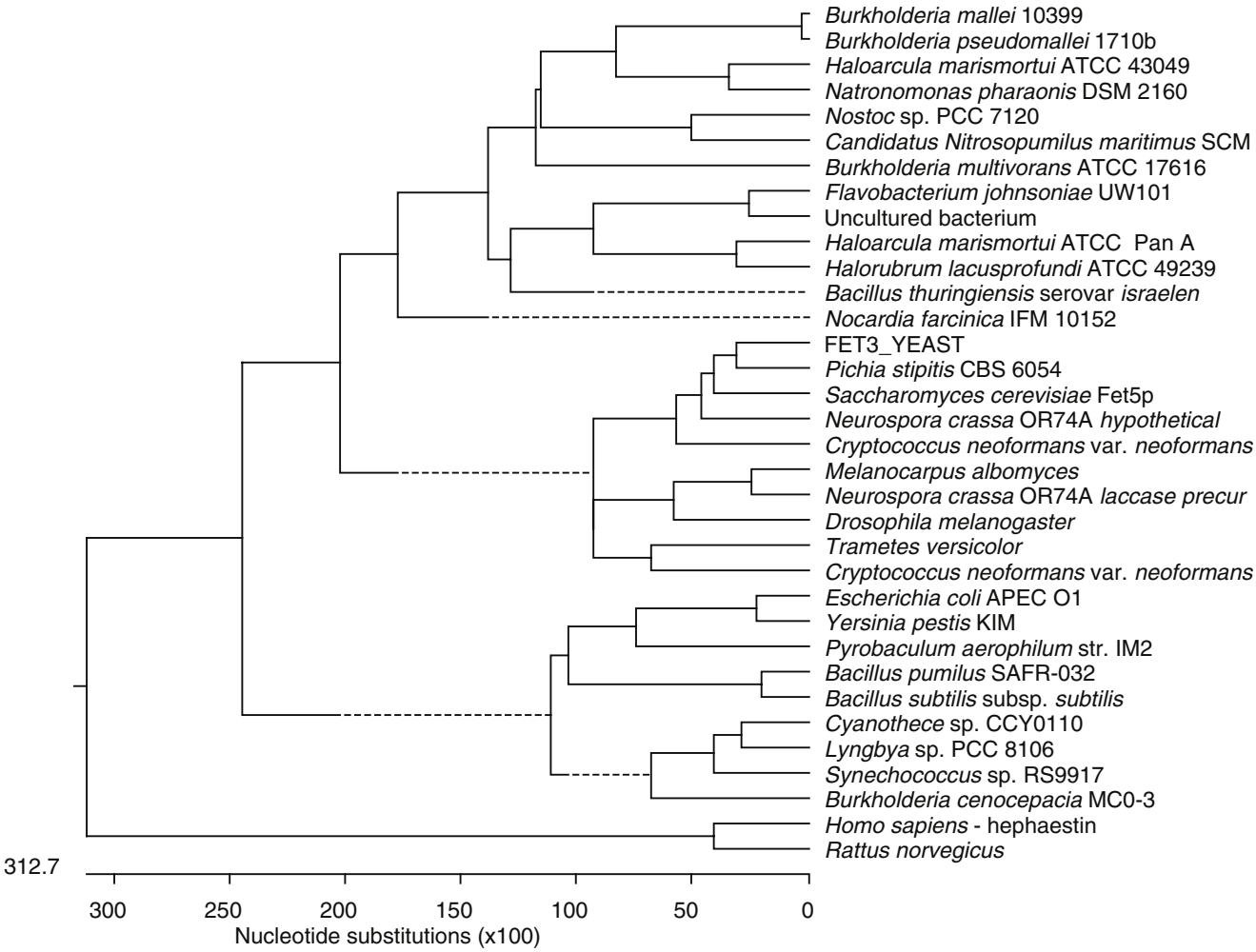

Fig. 1 Dendrogram constructed using multicopper oxidase from diverse archaeal, bacterial and eucaryal sources (http://img.jgi.doe.gov)

Table 2 Archaeal multicopper oxidase (putative laccase) sequence annotation using Pfam protein families database (http://pfam.sanger.ac.uk/)

Archaea	PfamA	AA	Entry type	Bits score	E value
P. aerophilum	Cu-oxidase 3	110	Domain	126.1	9.9e-35
	Cu-oxidase	147	Domain	-20.4	0.015
	Cu-oxidase 2	145	Domain	76.4	9.4e-20
C. Nitropumilus maritimus	Cu-oxidase 3	112	Domain	64.1	4.7e-16
	Cu-oxidase 2	117	Domain	5.7	0.00028
H. marismortui ATCC43049	Cu-oxidase 3	115	Domain	33.1	6.4e-10
H. marismortui ATCC43049 Pan1	Cu-oxidase 3	111	Domain	43.1	6.7e-09
	Cu-oxidase 2	125	Domain	61.8	2.3e-15
H. lacusprofundi ATCC49239	Cu-oxidase 3	111	Domain	58.9	1.7e-14
	Cu-oxidase 2	113	Domain	38.8	2e-08
N. pharaonis DSM2160	Cu-oxidase 3	115	Domain	17.2	2.7e-08

was comparable to fungal laccases (Table 2). Further, each input sequence the server predicts enzyme/non-enzyme and enzyme class. The score consists estimated probability and class/category to which the sequences belong (Table 3). Most of putative laccase sequences were found to be enzymes belonging to oxidoreductases. Even, few of them

Table 3 Prediction of enzyme class (putative laccases) from archaeal sources obtained from cluster of orthologous groups (http://www.cbs.dtu.dk/servises/ArchaeaFun/)

Archaea	Enzyme class	Enzyme class		Enzyme	
		Prob/odd		Prob/odd	
P. aerophilum	Oxidoreductase	0.247	1.112	0.597	1.194
	Transferase	0.190	0.855		
	Hydrolase	0.196	0.882		
C. Nitropumilus maritimus	Oxidoreductase	0.211	0.950	0.400	0.800
	Transferase	0.210	0.945		
	Hydrolase	0.271	1.220		
C. Nitropumilus maritimus	Oxidoreductase	0.234	1.053	0.420	0.840
	Transferase	0.176	0.792		
	Hydrolase	0.223	1.004		
H. marismortui ATCC43049	Oxidoreductase	0.178	0.801	0.576	1.152
	Transferase	0.186	0.837		
	Hydrolase	0.150	0.675		
H. marismortui ATCC43049 Pan1	Oxidoreductase	0.297	1.337	0.496	0.992
	Transferase	0.182	0.819		
	Hydrolase	0.177	0.797		
H. lacusprofundi ATCC49239	Oxidoreductase	0.228	1.026	0.601	1.202
	Transferase	0.154	0.693		
	Hydrolase	0.235	1.058		
N. pharaonis DSM2160	Oxidoreductase	0.202	0.909	0.610	1.220
	Transferase	0.154	0.693		
	Hydrolase	0.153	0.689		

were also found to be either transferase or hydrolases with marginal probability, which signifies its evolutionary primitiveness. This method relies on predicted proteins features like co-translational and post-translational modifications, secondary structure and simple physicochemical properties [14].

Archaeal two-domain multicopper blue proteins have not been previously studied. The BLAST programme was used to search the non-redundant (nr) set of protein sequences provided at NCBI (NIH, Bethesda). Sequence alignments around the copper binding sites of the novel two-domain archaeal multicopper blue proteins were studied (Fig. 2). Since the monomeric two-domain multicopper blue proteins cannot form interdomain copper binding sites, these proteins are presumed to aggregate to form homotrimers, like the case of nitrite reductase [16]. Domain alignment in our studies establishes archaeal MCOs as laccases (Fig. 2).

Contrary to the statement that the gene repertoire overlapped more with Euryarchaeota than with bacteria or eukarya [17], here we could interestingly find all the multicopper oxidases of phylum Euryarchaeota to be deeply rooted and in a separate clade whereas shallow rooted multicopper oxidase from Crenarchaeota. For example, *P. aerophilum* was found to be phylogenetically closer to eubacteria (Fig. 1). The exceptional archaeal features make the archaeal domain of life an interesting area of research for novel protein. Translated proteins of multicopper oxidase reported here is of very diverse properties, i.e. copper-containing nitrite reductase, membrane protein (Pan1), cupredoxin, oxidoreductases activity, multicopper oxidase type 2 and multicopper oxidase type 3.

Archaeal multicopper oxidase share the conspicuous copper-binding site pattern with membrane protein (Pan1a), prokaryotic azurins, multicopper oxidase type 3 and nitrite reductase (NirK) fit in perfectly as a common ancestral form of multicopper oxidase. Most of the multicopper oxidase reported here were of Type 1 and Type 2, whereas in *P. aerophilum* IM2 and *Halorubrum lacusprofundi* ATCC49239, the entire three copper-binding domain could be found. An analysis of the genome sequence database revealed novel types of two-domain laccases. These two-domain proteins have a conspicuous combination of blue-copper and interdomain trinuclear copper binding residues, which is common in nitrite reductase, ceruloplasmin and

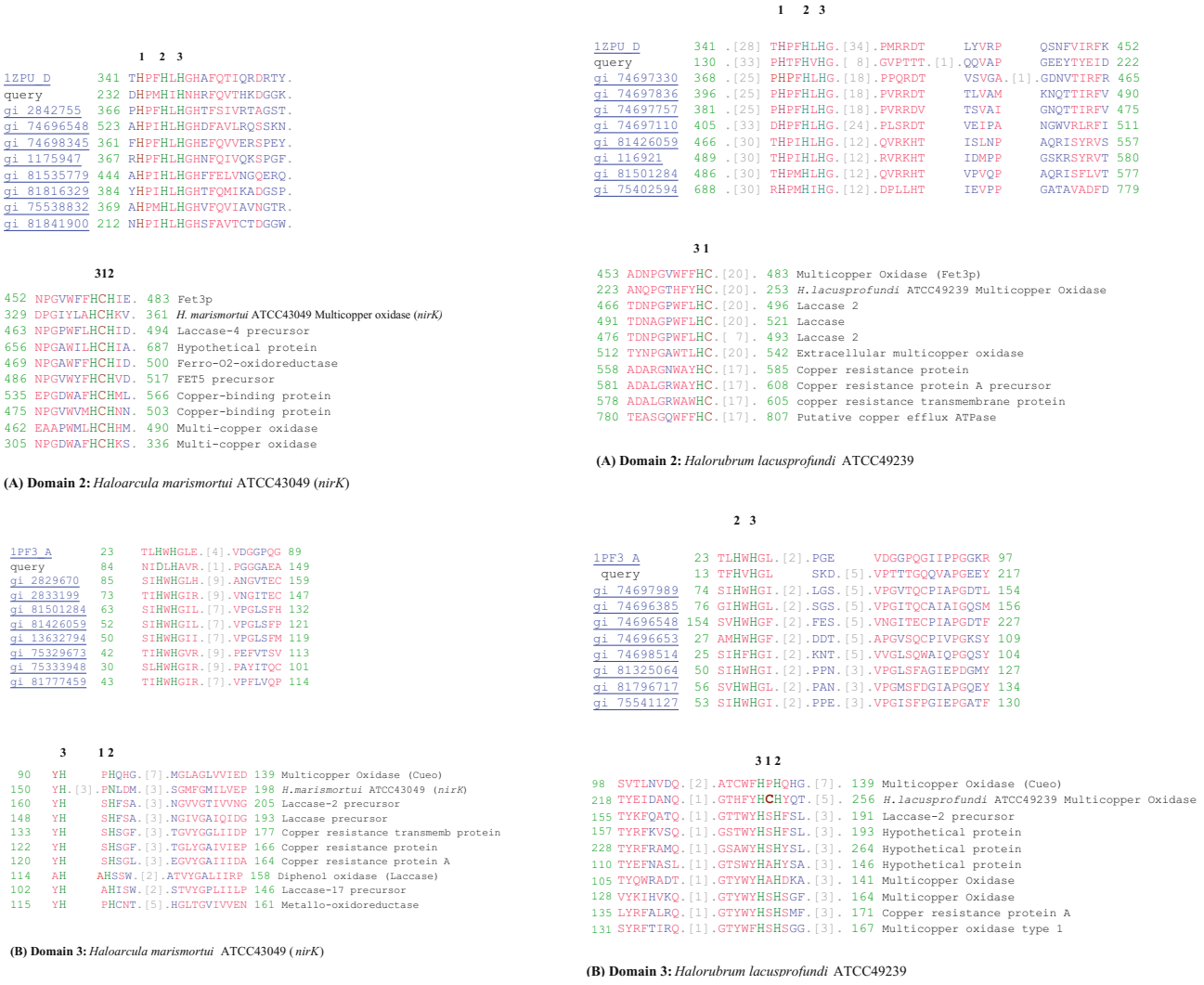

Fig. 2 Sequence alignment around the copper-binding (A). Domain 2; (B). Domain 3, in multicopper blue proteins of *Haloarcula marismortui* ATCC43049 (*nirK*) and *Halorubrum lacusprofundi* ATCC49239. The numbers 1, 2 and 3 at the top of the alignments, indicate the consensus positions of the type 1, type 2 and type 3 copper-binding residues, respectively. The residue colored in brown is of BCB sites. The residues colored in green are type 2/3 IDCB sites.

ascorbate oxidase (Fig. 2). So it can be presumed that the archaeal laccases might be the plausible ancestral form of nitrite reductase, ceruloplasmin and ascorbate oxidase.

Conclusion

Our analysis strongly suggests that laccases could be obtained from archaeal sources with robust biocatalytic functions. Moreover, it opens up broad opportunities for novel biocatalyst with broad biotechnological applications. New functions of the multicopper oxidase family may emerge from the novel proteins we have identified in diverse archaeal species. Moreover, the biological functions of these proteins should be revealed by future laboratory experiments.

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