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Diversity of the Heme–Copper Superfamily in Archaea: Insights from Genomics and Structural Modeling

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Abstract Recent advances in DNA sequencing technologies have provided unprecedented access into the diversity of the microbial world. Herein we use the comparative genomic analysis of microbial genomes and environmental metagenomes coupled with structural modelling to explore the diversity of aerobic respiration in Archaea. We focus on the heme–copper oxidoreductase superfamily which is responsible for catalyzing the terminal reaction in aerobic respiration—the reduction of molecular oxygen to water. Sequence analyses demonstrate that there are at least eight heme–copper oxygen reductase families: A-, B-, C-, D-, E-, F-, G-, and H-families. Interestingly, five of these oxygen reductase families (D-, E-, F-, G-, and H-families) are currently found exclusively in Archaea. We review the structural properties of all eight families focusing on the members found within Archaea. Structural modelling coupled with sequence analysis suggests that many of the oxygen reductases identified from thermophilic Archaea have modified proton channel properties compared to the currently studied mesophilic bacterial oxygen reductases. These structural differences may be due to adaptation to the specific environments in which these enzymes function. We conclude with a brief analysis of the phylogenetic distribution and evolution of Archaeal heme–copper oxygen reductases.

1 Introduction

All life on Earth is currently divided into three Domains based on phylogenetic and genomic analyses: Bacteria, Archaea, and Eukaryota (Woese and Fox 1977). Early work on Archaea characterized them as predominantly inhabiting extreme environments (hyperthermophiles, halophiles, acidophiles) or as performing novel metabolisms (methanogens). Environmental sampling utilizing rDNA as a phylogenetic marker has revealed an enormous diversity of microbial life (DeLong and Pace 2001; DeSantis et al. 2006), with the vast majority belonging to uncultured species (Rappe and Giovannoni 2003). Recently, it has become apparent that Archaea are not limited to extreme environments, but are common in almost every environment studied (Robertson et al. 2005). In fact the diversity of Archaea is greatest in cold envi-

ronments, where they form a significant fraction of the biosphere (Cavicchioli 2006). However, very little is known about the physiology and ecology of these new Archaeal groups, creating a fascinating opportunity for the characterization of the bioenergetic properties of these organisms.

Recent advancements in DNA sequencing and analysis applied to microbial genomes and communities (Riesenfeld et al. 2004) have vastly improved our understanding of microbial diversity and physiology. Herein, we review the comparative genomics and structural diversity of the heme–copper superfamily in Archaea. Members of the superfamily play key roles in aerobic and anaerobic respiration. Analysis of the DNA sequences from more than 950 genomes from Archaea and Bacteria along with 15 metagenomic projects has identified thousands of new members of the heme–copper superfamily. The superfamily can currently be classified into eight oxygen reductase and five nitric oxide reductase families. It is very interesting that five of the oxygen reductase families are currently only found in Archaea. This leads to questions concerning the evolution and ecology of Archaeal members of the superfamily.

2 Introduction to the Heme–Copper Superfamily

The majority of Eukaryota are aerobic heterotrophs, oxidizing organic compounds to CO_2 while reducing O_2 to water via aerobic respiration within mitochondria. Archaea and Bacteria have much greater physiological diversity, extracting energy from their environments utilizing a wide range of metabolisms including; aerobic and anaerobic respirations, fermentation, and photosynthesis (Madigan and Martinko 2006). In aerobic eukaryotes, respiration is carried out within the inner mitochondrial membrane by a linear electron transfer chain with cytochrome c oxidase as the terminal electron acceptor. In Archaea and Bacteria, respiration is performed by a series of soluble and integral membrane protein complexes found within cytoplasmic or thylakoid membranes (Madigan and Martinko 2006). Archaea and Bacteria usually have complex branched respiratory chains with alternative terminal electron acceptors (Poole and Cook 2000) (aerobic and anaerobic), allowing them to adjust their metabolisms to fit the availability of substrates in their environments.

The heme–copper superfamily is a large and extremely diverse superfamily of integral membrane proteins that contains members which play crucial roles in both aerobic and anaerobic respiration (Garcia-Horsman et al. 1994; Pereira et al. 2001). Members of the superfamily are oxidoreductases, which currently are divided into two classes based on the chemical reaction that they perform: the oxygen reductases and the nitric oxide reductases.

Oxygen reductases $\quad O_2 + 4H_{chem}{}^+ + 4e^- + 4H_{in}{}^+ \rightarrow 2H_2O + 4H_{out}{}^+$,

Nitric oxide reductases $\quad 2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$.

The oxygen reductase members of the superfamily are terminal oxidases in the aerobic respiratory chains of mitochondria and many Archaea and Bacteria. These enzymes catalyze the reduction of molecular oxygen to water with the concomitant transfer of protons across the membrane, contributing to the generation of the electrochemical gradient used for membrane transport and ATP synthesis (Michel et al. 1998; Hosler et al. 2006). The oxygen reductases have a broad phylogenetic distribution and are found in all three domains of life: Archaea, Bacteria, and Eukaryotes. The nitric oxide reductase (NOR) members of the superfamily catalyze the reduction of nitric oxide to nitrous oxide in Archaea and Bacteria capable of anaerobic denitrification starting with nitrate or nitrite (Zumft 2005). Nitric oxide reductase can also play a nitric oxide detoxifying role in some pathogenic bacteria (Philippot 2005). Unlike the oxygen reductases, the currently identified nitric oxide reductases are not electrogenic and are unable to pump protons (Reimann et al. 2007). Nitric oxide reductases have only been found in Archaea and Bacteria, however recently the unicellular eukaryotic foraminifer *Globobulimina pseudospinescens* has been shown to perform denitrification (Risgaard-Petersen et al. 2006). The genes responsible for denitrification in this organism have not been characterized, so it is not clear whether a heme–copper nitric oxide reductase or an alternative nitric oxide reducing enzyme performs the nitric oxide reduction step.

Structurally, both the oxygen and nitric oxide reductases are multisubunit, integral membrane protein complexes. The complexes are composed of a main subunit, which is the functional core of the enzyme complex, along with secondary subunits that serve a variety of functions, such as interacting with the mobile electron donors (e.g., cytochrome c or quinol) or regulatory functions. The main subunit (subunit I) contains all of the amino acids and cofactors necessary for both catalysis and proton translocation and it is this core subunit that defines the superfamily (Garcia-Horsman et al. 1994). Only this subunit is homologous between the oxygen and nitric oxide reductases, with the secondary subunits having independent evolutionary histories.

3 Classification of the Superfamily

We have analyzed DNA sequences from microbial genomes and metagenomic projects to identify thousands of new members of the heme–copper superfamily (Hemp 2007). To characterize this diversity we proposed an updated classification system in which the superfamily is first divided into classes, and then further into families, and subfamilies (see Pereira et al. 2001 for the original classification scheme). Classes are defined by the reaction that the enzymes catalyze. The superfamily is currently divided into two classes: the oxygen reductases and the nitric oxide reductases. It is possible that some enzymes of one class may also have low rates of reaction for the other class

(Giuffre et al. 1999). In these cases enzymes were placed into classes based on the primary reaction that they catalyze. It should be noted that the class is not a phylogenetic property in that the reaction types could have evolved independently multiple times within the superfamily.

Families were delineated based on phylogenetic, genomic, sequence, and structural information. Members of a given family were defined to perform the same chemical reaction, share common protein complex subunit architecture, have similar proton channel structures, and form separate sequence clusters. Different families clearly have different physiological functions, are paralogous to each other, and can be objectively defined by sequence analyses. Phylogenetic analysis of the whole superfamily using Bayesian (Ronquist and Huelsenbeck 2003), Neighbor-Joining, and Maximum Parsimony methods all produced 13 phylogenetic clusters irrespective of the method used. The same 13 families were identified using all-vs-all BLAST sequence clustering with a 35% sequence identity cutoff. The intra-family sequence identity was at least 35%, whereas inter-family sequence identities were usually less than 20%. This shows that the differences between families is quite large. Structural analysis (active-site structure and channel properties) and subunit architecture further support the delineation of the currently identified superfamily members into 13 families.

Table 1 Family properties of heme–copper superfamily members

Family	Class	Active-site residue	Cross-linked cofactor	Proton channels[a]	Proton pumping	Subunit II fold[b]
A	Oxygen reductase	Y	+	2[c]	+	Cu_A
B	Oxygen reductase	Y	+	1	+	Cu_A
C	Oxygen reductase	Y	+	1	+	Heme c
D	Oxygen reductase	Y	+*	1	+	Other
E	Oxygen reductase	Y	+*	1	+	Cu_A
F	Oxygen reductase*	Y	+*	0	–*	Cu_A
G	Oxygen reductase*	Y	+*	1	+*	Cu_A
H	Oxygen reductase*	Y	+*	1	+*	Cu_A
cNOR	Nitric oxide reductase	E	–	0	–	Heme c
qNOR	Nitric oxide reductase	E	–	0	–	–
sNOR	Nitric oxide reductase*	N	–	0	–*	Cu_A
eNOR	Nitric oxide reductase*	Q	–	1	+/–	Cu_A
gNOR	Nitric oxide reductase*	D	–	0	–*	Cu_A

[a] Number of conserved proton channels determined by sequence analysis and structural modelling

[b] Cu_A-Subunit II has cupredoxin fold, homologous to subunit II of mitochondrial cytochrome c oxidases. Heme c-Subunit II has cytochrome c fold

[c] Some members of the A-family have modified D- and/or K-channels

* Predicted

Eight of the families identified belong to the oxygen reductase class: the A-, B-, C-, D-, E-, F-, G-, and H-type oxygen reductases. Two of the families belong to the nitric oxide reductase class: the cNOR and qNOR nitric oxide reductases. The other three families have not been previously described. On the basis of expression data (Cho et al. 2006) and structural analysis we predict that all three families belong to the nitric oxide reductase class: putatively the sNOR, eNOR, and gNOR nitric oxide reductases. Table 1 lists the heme–copper families along with their associated properties.

4
Heme-Copper Family Properties

Many of the recently identified oxygen reductase families are currently only found in Archaea: the D-, E-, F-, G-, and H-families. These new families have unique structural features in comparison to the previously studied A-, B-, and C-families. It is also common for Archaea to contain members of the A- and B-families that have modified structural features. The reason that Archaea have modified heme–copper superfamily members is not yet clear. It may be related to the structural properties of their membranes, or to the environments in which they are found. To identify the structural features that are modified in Archaea we first review the structural properties of oxygen reductases already elucidated from the study of bacterial enzymes.

4.1
Oxygen Reductases

The oxygen reductases are structurally diverse. They vary in the type of electron donor (cytochrome c, quinol, or high potential iron-sulfur protein), the types of heme present [hemes B, O, O_{P1}, O_{P2}, A, or A_S (Lübben and Morand 1994)], and the number of subunits present in the enzymatic complex (ranging from 2, in some bacteria, to as many as 13 in mammalian mitochondria). X-ray crystal structures have been reported for members of both the A-type (Ostermeier et al. 1997; Abramson et al. 2000; Yoshikawa et al. 2000; Svensson-Ek et al. 2002) and B-type (Soulimane et al. 2000) oxygen reductase families. These structures show that the protein component of the main subunit, subunit I, has 12 transmembrane helices arranged in a pseudo three-fold rotational symmetry with the symmetry axis perpendicular to the membrane. Subunit I also contains three redox-active metals; a low-spin heme and a binuclear heme–copper catalytic site. The six-coordinate low-spin heme accepts electrons from the electron donor specific for the particular complex and transfers the electrons to the catalytic site. The heme–copper catalytic site is composed of a five coordinate high-spin heme and a copper ion ligated to three conserved histidines. In three of the oxygen reductase families (A-, B-, and C-families) a novel His-Tyr cross-

linked cofactor has been shown by X-ray crystallography (A- and B-families) or mass spectrometry (A-, B-, and C-families) (Hemp et al. 2006; Rauhamaki et al. 2006) to be present in the active site. This cross-linked pair of residues forms a cofactor which plays a critical role in oxygen reduction. Since the other five families (D-, E-, F-, G-, and H-families) conserve the residues which form the cross-link it is likely that they also contain this cofactor.

Mutagenesis studies performed on bacterial oxygen reductase members of the superfamily have identified proton input channels necessary for proton pumping and the delivery of protons from the cytoplasm to the active site. In the A-type oxygen reductases, two proton input channels have been identified (Hosler et al. 1993). One channel, the K-channel, leads from the interface of subunits I and II on the cytoplasmic side of the membrane (equivalent to the matrix side of the mitochondrial enzymes) to the cross-linked tyrosine at the active-site. The K-channel has been shown to be important in the A-type oxidases for the delivery of chemical protons to the catalytic active site (Konstantinov et al. 1997). The second channel, the D-channel, has been implicated in the transfer of both chemical protons to the active site and protons which are pumped across the membrane. The D-channel leads from an aspartate on the cytoplasmic surface of subunit I to a gating residue near the active site which is a branch point from which protons are directed either to the active site, where they are consumed to generate water, or are pumped across the membrane and released on the opposite side as pumped protons, contributing to the electrochemical gradient. Both the K- and D-channels contain highly conserved hydrophilic residues which help to stabilize and orient the water in conformations facilitating proton transfer (Wraight 2006).

In the B-type and C-type oxygen reductases, a channel analogous to the K-channel has been identified through modelling studies (B- and C-families) (Sharma et al. 2006; Hemp et al. 2007), X-ray crystallography (B-family) (Soulimane et al. 2000) or site-directed mutagenesis (C-family) (Hemp et al. 2007). The X-ray structure of the ba_3-type oxidase (B-family) from *Thermus thermophilus* has been used to postulate two additional proton input channels, one analogous to the D-channel, and another called the Q-channel (Soulimane et al. 2000). There are no experimental studies to support a functional role of these putative channels, and sequence analysis indicates that the D- and Q-channels are not conserved in the B-family of oxygen reductases. Similar analyses indicates that there is only one conserved channel in the C-family (Hemp et al. 2007). Hence, it is highly likely that there is only one proton input channel in the B- and C-families of oxidases.

4.2 Nitric Oxide Reductases

The nitric oxide reductases are also structurally diverse and vary in the number of subunits present and the type of electron donor. There are currently

two established families (Hendriks et al. 2000; Zumft 2005) cNOR and qNOR, and our recent work indicates three additional putative families of nitric oxide reductases: sNOR, eNOR, and gNOR families (Stein et al. 2007; Sievert et al. 2007). All five nitric oxide reductase families have a core subunit that is homologous to the main subunit in the oxygen reductases. The cNOR family forms a two-subunit complex. The main subunit (NorB) is homologous to the core subunit in the oxygen reductases and contains the catalytic site, whereas the second subunit (NorC) contains heme c and acts as an electron shuttle between a mobile cytochrome c and the core subunit. The enzymes in the qNOR family contain a single protein subunit (NorZ), which appears to be a fusion of the core subunit and a second subunit (Cramm et al. 1997). The qNOR's do not contain cytochrome c nor use cytochrome c as an electron donor. Instead, the qNOR enzymes receive electrons from membrane-bound quinols. There have been reports of a hybrid type of nitric oxide reductase which can receive electrons from either quinol or cytochrome c (Suharti et al. 2001). However, no sequence data is available to allow this enzyme to be classified.

There are no X-ray structures of a nitric oxide reductase. However, the nitric oxide reductases are homologous to the oxygen reductases and homology modelling coupled with spectroscopic data and metal analysis has allowed structural models to be inferred (Zumft 2005; Reimann et al. 2007). In the cNOR and qNOR families the main subunit (NorB or NorZ, respectively) contains a six-coordinate low spin heme, which accepts electrons from the electron donor (cytochrome c or quinol, respectively), and a binuclear active site, where catalysis occurs. The binuclear center in the nitric oxide reductases does not contain copper, as in the oxygen reductases, but instead contains an iron ion (Girsch and de Vries 1997; Hendriks et al. 1998). The three conserved histidine residues which ligate to Cu_B in the active site of the oxygen reductases are also present in the NO reductases. However, the ligation state of the iron is not clear, electronic structure calculations (Blomberg et al. 2006) and small molecule active-site models (Collman et al. 2006) suggest that a conserved glutamate acts as a fourth ligand to the iron, whereas in whole protein models it appears only the three histidine ligands are present (Zumft 2005; Reimann et al. 2007). The NO reductases do not have the His-Tyr cross-linked cofactor in the active site and do not perform oxygen reduction at an appreciable rate. It appears that all the members of the heme–copper superfamily that contain the His-Tyr cofactor are oxygen reductases whereas those without it are nitric oxide reductases.

The cNOR and qNOR families of nitric oxide reductases are not electrogenic, i.e., charges do not cross the membrane bilayer concomitant with enzyme catalysis (Reimann et al. 2007). The protons required for chemistry are delivered from the periplasmic side of the membrane and not, as in the case of the oxygen reductases, from the cytoplasmic side. Hence, there is no need for proton input channels from the cytoplasmic side of the enzymes. Indeed, there is no pattern of conservation of residues that define any proton

channel from the cytoplasmic surface of the NO reductases (Reimann et al. 2007). Instead a proton input channel that leads to the active site, and which contains a pair of conserved glutamate residues near the periplasmic surface (Reimann et al. 2007; Thorndycroft et al. 2007), has been postulated.

5
Heme–Copper Oxygen Reductases in Archaea

There are many excellent reviews covering the biochemical properties of heme–copper oxygen reductases in Archaea (Schäfer et al. 1999; Schafer 2004), so we will focus on the structural properties determined by sequence analysis coupled with structural modelling. These analyses demonstrate that the Archaea harbor seven different families of heme–copper oxygen reductases, many of which have only one conserved proton channel (Tables 2 and 3). Comparative studies of these oxidases will be crucial to determine the mechanism of proton pumping.

5.1
A-Family

The A-family is the largest and best studied heme–copper family. The enzyme complex typically contains three main subunits, subunits I, II, and III, which are homologues of the subunits of the prototypical A-family oxidase, the mitochondrial cytochrome c oxidase. Many of the A-family complexes contain additional subunits (Fig. 1A). Homologues of subunit III are only found in the A-family oxygen reductases. In Archaea it is common for subunit III to be fused to the C-terminus of subunit I. On the basis of sequence divergence, approximately 20 subfamilies have been defined within the A-family of oxygen reductases. Of these 20 subfamilies, seven are exclusively found in Archaea. There are also a number of unclassified A-family oxidases identified within Archaea. These are identified in Table 3.

The most-studied A-family member from Archaea is the SoxM supercomplex from *Sulfolobus acidocaldarius* (Schafer 2004), listed as Subfamily XIV in Table 2. The SoxM supercomplex from *Sulfolobus acidocaldarius* is a fusion of an Archaeal bc_1 homolog (SoxFG) and an A-type oxygen reductase (SoxM) (Komorowski et al. 2002). The SoxFG bc_1 homolog oxidizes caldariella quinol and transfers the electrons via sulfocyanin (SoxE) to Cu_A, which is located in subunit II of the oxygen reductase. The core subunit of SoxM is a fusion of subunits I+III, and is unique in that the active site contains heme b. This is the only known example of a heme b incorporated into an A-type oxygen reductase active-site, forming a heme b_3-Cu_B binuclear center. A-family oxygen reductases have also been isolated in *Metallosphaera sedula* (Kappler et al. 2005), *Aeropyrum pernix* (Ishikawa et al. 2002), *Pyrobaculum oguniense*

Table 2 Phylogenetic distribution of Archaeal heme–copper and bd oxidase superfamily members. Organisms are classified by domain, phylum, class, and order. Organisms in *bold* are from completed genomes, those in *plain font* are from incomplete genomes, while those in italics are from individual submissions to GenBank. The number of heme–copper and bd oxidase members from each family that an organism has is given in the respective column. bd oxidases *underlined* have two subunit I genes encoded instead of subunits I and II

	bd	A	B	C	D	E	F	G	H	qNOR	eNOR
Crenarchaeota											
Thermoprotei											
Caldisphaerales											
Cenarchaeales											
Cenarchaeum symbiosum		1									
Desulfurococcales											
Aeropyrum pernix (K1)		1	1								
Hyperthermus butylicus (DSM 5456)	1										
Ignicoccus (Kin4-I)											
Staphylothermus marinus (F1)											
Marine archaeal group 1											
Nitrosopumilus maritimus (SCM1)		1									
Sulfolobales											
Acidianus ambivalens					1						
Metallosphaera sedula (DSM 5348)		1			2	1	1				
Sulfolobus acidocaldarius (DSM 639)		1			1	1					
Sulfolobus metallicus							1				
Sulfolobus solfataricus (P2/ATCC 35092/DSM 1617)		1			1	1				1	
Sulfolobus tokodaii (7/JCM 10545)		1			1	1	1				
Thermoproteales											
Caldivirga maquilingensis (IC-167)	1							1		1	
Pyrobaculum aerophilum (IM2/ATCC 51768/DSM 7523)		1	1							1	

Table 2 (continued)

	bd	A	B	C	D	E	F	G	H	qNOR	eNOR
Pyrobaculum arsenaticum (9DSM 13514)	1									1	
Pyrobaculum calidifontis (JCM 11548)		1	1							1	
Pyrobaculum islandicum (DSM 4184)	1										
Pyrobaculum oguniense		1	1								
Thermofilum pendens (Hrk 5)	1										
Euryarchaeota											
Archaeoglobi											
Archaeoglobales											
Archaeoglobus fulgidus (DSM 4304/ATCC 49558)	1										
Halobacteria											
Halobacteriales											
Haloarcula marismortui (ATCC 43049)		2	1							1	
Halobacterium salinarum (NRC-1/ATCC 700922)	4	1	1								
Halobacterium salinarum (R1/ATCC 19700)	2	1	1								
Haloferax volcanii (DS2/ATCC 29605)	1	1	1							1	
Halorubrum lacusprofundi (ATCC 49239)		1	1							1	
Haloquadratum walsbyi (DSM 16790)		1									
Natronomonas pharaonis (DSM 2160)		1	1								1
Methanobacteria											
Methanobacteriales											
Methanosphaera stadtmanae (DSM 3091)											
Methanothermobacter thermautotrophicum (Delta H)											
Methanococci											
Methanococcales											
Methanocaldococcus jannaschii (DSM 2661/ATCC 43067)											
Methanococcus aeolicus (Nankai-3)											

Table 2 (continued)

	bd	A	B	C	D	E	F	G	H	qNOR	eNOR
Methanococcus maripaludis (C5)											
Methanococcus maripaludis (C7)											
Methanococcus maripaludis (S2/LL)											
Methanococcus vannielii (SB)											
Methanomicrobia											
Methanomicrobiales											
Methanobrevibacter smithii (ATCC 35061)											
Methanocorpusculum labreanum (Z)											
Methanoculleus marisnigri (JR1)											
Methanoregula boonei (6A8)											
Methanospirillum hungateii (JF-1)											
Methanosarcinales											
Methanococcoides burtonii (DSM 6242)											
Methanosaeta thermophila (PT/DSM 6194)											
Methanosarcina acetivorans (C2A/ATCC 35395/DSM 2834)	1										
Methanosarcina barkeri (fusaro)	1										
Methanosarcina mazei (Go1/ATCC BAA-199/DSM 3647)											
Methanopyri											
Methanopyrales											
Methanopyrus kandleri (AV19/DSM 6324/JCM 9639)											
Thermococci											
Thermococcales											
Pyrococcus abyssi (GE5/Orsay)											
Pyrococcus furiosus (DSM 3638/ATCC 43587)											
Pyrococcus horikoshii (OT3)											
Thermococcus kodakaraensis (KOD1)											

Table 2 (continued)

	bd	A	B	C	D	E	F	G	H	qNOR	eNOR
Thermoplasmata											
Thermoplasmatales											
Ferroplasma (type II)		**1**									
Ferroplasma acidarmanus (fer1)	**2**	**1**									
Picrophilus torridus (DSM 9790/ATCC 70027)	**2**	**1**									
Thermoplasma acidophilum (DSM 1728)	**2**										
Thermoplasma volcanium (GSS1/DSM 4299/JCM 9571)	**2**										
Thermoplasmatales archaeon (Gpl)	**2**								**1**		
Unclassified											
Methanogenic archaea (Rice Cluster I)											
Korarchaeota											
Nanoarchaeota											
Nanoarchaeum											
Nanoarchaeum equitans (Kin4-M)											

Table 3 Archaeal members of the heme–copper superfamily. This table lists all of the currently known members of the heme–copper superfamily found within Archaea, including those from environmental samples. The organism, identification number, operon structure, and type of electron acceptor (subunit II) are given for each member. The NCBI identification number is given for environmental samples

A-family oxygen reductases			
Subfamily XII			
Aeropyrum pernix (K1)	5010020	II I+III Sco1	Cu_A
Subfamily XIII			
Haloarcula marismortui (ATCC 43049) [1]	5020319a	III [I] II	Cu_A
Haloarcula marismortui (ATCC 43049) [3]	5020319c	[II] [I] [III]	Cu_A
Halobacterium salinarum (NRC-1/ATCC 700922)	5020320	II [III] I	Cu_A
Halobacterium salinarum (R1/ATCC 19700) [1]	5020321	II I III	Cu_A
Haloferax volcanii (DS2/ATCC 29605) [2]	5020322b	[II CtaB] [I] [III]	Cu_A
Haloquadratum walsbyi (DSM 16790)	5020323	[II CtaB] [I] [III]	Cu_A
Natronomonas pharaonis (DSM 2160)	5020447	CtaB II III I	Cu_A
Subfamily XIV			
Metallosphaera sedula (DSM 5348)	5010392	[SoxF SoxG II SoxI] SoxE I+III	Cu_A
Sulfolobus acidocaldarius (DSM 639)	5010718	[SoxF SoxG II SoxI] SoxE I+III	Cu_A
Sulfolobus solfataricus (P2/ATCC 35092/DSM 1617)	5010719	[SoxF SoxG II SoxI] SoxE I+III	Cu_A
Sulfolobus tokodaii (7/JCM 10545)	5010720	[SoxF SoxG II SoxI] SoxE I+III	Cu_A
Subfamily XV			
Pyrobaculum aerophilum (IM2/ATCC 51768)	5010543	II I+III X CtaB	Cu_A
Pyrobaculum calidifontis (JCM 11548)	5010893	II I+III X CtaB	Cu_A
Pyrobaculum oguniense	501X004	II I+III	Cu_A
Subfamily XVI			
Ferroplasma (type II)	5020274	I+III Sulfocyanin II CtaB	Cu_A
Ferroplasma acidarmanus (fer1)	5020275	I+III Sulfocyanin II CtaB	Cu_A
Picrophilus torridus (DSM 9790/ATCC 70027)	5020496	I+III Sulfocyanin II CtaB	Cu_A

Table 3 (continued)

Subfamily XVII			
Cenarchaeum symbiosum	5010160	X II I Cyanin CtaA	Cu_A
Nitrosopumilus maritimus (SCM1)	5011037	X II I Cyanin CtaA	Cu_A
	AACY020042774		
	AACY020165265		
	AACY020271526		
	AACY020383948		
	AACY020408154		
	AACY021781753		
	AACY022062082		
	AACY022206108		
	AACY022690326		
	AACY023085447		
	AACY023776262		
	AACY024074640		
Subfamily XVIII			
Haloarcula marismortui (ATCC 43049) [2]	5020319b	II I+III IV	Cu_A
Haloferax volcanii (DS2/ATCC 29605) [1]	5020322a	II I+III IV	Cu_A
Halorubrum lacusprofundi (ATCC 49239)	5020995	II I+III IV	Cu_A
Unclassified subfamily			
Euryarchaeota (Predicted from genomic context)	AACY01004662	II I III	Cu_A
	AACY020067637		
	AACY020071257		
	AACY020098741		
	AACY020109206		
	AACY020112219		
	AACY020116897		
	AACY020207114		

Table 3 (continued)

AACY020242961
AACY020255478
AACY020321485
AACY020327424
AACY020330951
AACY020335240
AACY020338338
AACY020402825
AACY020499439
AACY020546099
AACY020561279
AACY021070130
AACY022154585
AACY022225003
AACY022384987
AACY022690422
AACY022694601
AACY022699076
AACY022700678
AACY023388866
AACY023397990
AACY023399007
AACY023410494
AACY023454892
AACY023474211
AACY023519752
AACY023526653
AACY024102295

Table 3 (continued)

	DU733775		
B-family oxygen reductases			
Subfamily IV			
Haloarcula marismortui (ATCC 43049)	5020319	CbaE Halocyanin/IIa II I	Cu_A
Halobacterium salinarum (NRC-1/ATCC 700922)	5020320	CbaE Halocyanin/IIa II I	Cu_A
Halobacterium salinarum (R1/ATCC 19700)	5020321	CbaE Halocyanin/IIa II I	Cu_A
Haloferax volcanii (DS2/ATCC 29605)	5020322	CbaE IIa II I	Cu_A
Halorubrum lacusprofundi (ATCC 49239)	5020995	CbaE IIa II I	Cu_A
Natronomonas pharaonis (DSM 2160)	5020447	CbaE IIa II I	Cu_A
Subfamily V			
Aeropyrum pernix (K1)	5010020	[IIa II sco1] [I]	Cu_A
Pyrobaculum aerophilum (IM2/ATCC 51768)	5010543	IIa II I sco1	Cu_A
Pyrobaculum calidifontis (JCM 11548)	5010893	IIa II I sco1	Cu_A
Pyrobaculum oguniense	501X004	IIa II I sco1	Cu_A
D-family oxygen reductases			
Acidianus ambivalens	501X001	doxB doxC doxE	quinol
Metallosphaera sedula (DSM 5348) [1]	5010392	doxB doxC doxE	quinol
Sulfolobus acidocaldarius (DSM 639)	5010718	doxB doxC doxE	quinol
Sulfolobus solfataricus (P2/ATCC 35092/DSM 1617)	5010719	doxB doxC doxE	quinol
Sulfolobus tokodaii (7/JCM 10545)	5010720	doxB doxC doxE	quinol
Operon code: doxB – subunit I homolog doxC – subunit II homolog (very distant)			
E-family oxygen reductases			
Subfamily I			
Metallosphaera sedula (DSM 5348)	5010392	soxA soxB soxC soxD	No Cu_A
Sulfolobus acidocaldarius (DSM 639)	5010718	soxA soxB soxC soxD	No Cu_A

Table 3 (continued)

Sulfolobus solfataricus (P2/ATCC 35092/DSM 1617)	5010719	soxA soxB soxC soxD	No Cu_A
Sulfolobus tokodaii (7/JCM 10545) [1]	5010720a	soxA soxB soxC soxD	No Cu_A
Subfamily II			
Sulfolobus tokodaii (7/JCM 10545) [2]	5010720b	soxA soxB	Cu_A
Operon code: soxB – subunit I homolog soxA – subunit II homolog			
F-family oxygen reductases			
Metallosphaera sedula (DSM 5348) [1]	5010392a	foxB foxC foxD foxE foxF foxJ foxG foxH [foxI foxA foxA]	Cu_A
Metallosphaera sedula (DSM 5348) [2]	5010392b		
Sulfolobus metallicus	501X002	foxG foxH foxI foxJ foxE foxF foxA [foxB foxC foxD]	Cu_A
Sulfolobus tokodaii (7/JCM 10545)	5010720	foxA foxB foxC foxD foxE foxF [foxG foxH foxI foxJ]	Cu_A
G-family oxygen reductases			
Caldivirga maquilingensis (IC-167)	5010896	[II soxE] I bc1	Cu_A
H-family oxygen reductases			
Thermoplasmatales archaeon (Gpl)	5020905	II I CtaB	Cu_A
qNOR-family nitric oxide reductases			
Caldivirga maquilingensis (IC-167)	5010896		
Haloarcula marismortui (ATCC 43049)	5020319		
Haloferax volcanii (DS2/ATCC 29605)	5020322		
Halorubrum lacusprofundi (ATCC 49239)	5020995		
Pyrobaculum aerophilum (IM2/ATCC 51768/DSM 7523)	5010543		

Table 3 (continued)

Pyrobaculum arsenaticum (9DSM 13514)	5010940		
Pyrobaculum calidifontis (JCM 11548)	5010893		
Sulfolobus solfataricus (P2/ATCC 35092/DSM 1617)	5010719		
eNOR-family nitric oxide reductases			
Natronomonas pharaonis (DSM 2160)	5020447	IIa II I	Cu_A

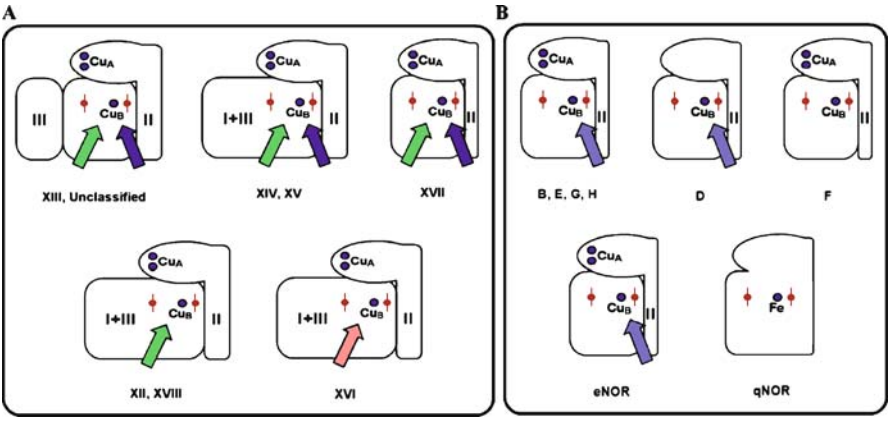


Fig. 1 Core subunit architecture of the heme–copper superfamily. **A** The structural diversity within the A-family in Archaea. **B** The other oxygen reductase and nitric oxide reductase families found in Archaea. Conserved channels are indicated by *arrows*. The K-channel is *blue*. The D-channel is *green*, while the modified D-channels are shown in *red*. The family and subfamily identifications are given in Table 3

(Nunoura et al. 2005), *Halobacterium halobium* (Fujiwara et al. 1989), and *Haloferax volcanii* (Tanaka et al. 2002).

The prototypical A-type oxygen reductases have two conserved proton channels, the D- and K-channels, for the transport of chemical and pumped protons. However, a few subfamilies that are unique to Archaea have modified D- or K-channels. Table 4 shows the key conserved residues that define these two channels. In three subfamilies (XII, XVI, and XVIII) most of the residues defining the K-channel are not conserved (Fig. 2), and in Subfamily XVII two of the residues are replaced by residues that could conceivably also function to assist proton transfer (e.g., *R. sphaeroides* K362 is replaced by a histidine). Subfamily XVI, in addition to missing conserved residues in the K-channel, has a modified D-channel in which one of the conserved asparagines (N207) is replaced by an aspartate (Table 4). The N207D mutant of the *R. sphaeroides* oxidase has been shown to be decoupled from the proton pump (Han et al. 2006). Therefore, the properties of this oxidase are clearly of interest. Figure 1A summarizes the features of the channels in the Archaeal A-family oxygen reductases.

5.2 B-Family

The B-type oxygen reductase complex consists of three subunits (Subunits I, II, and IIa). Subunits I and II are homologous to subunits I and II of the A-type family. The third subunit (IIa) is a single transmembrane helix which is structurally analogous to the second transmembrane helix in subunit II

Table 4 Channel properties of Archaeal heme–copper superfamily members. Conserved channel residues from known crystal structures (*Rhodobacter sphaeroides* [1M56] and *Thermus thermophilus* [1XME]) are given. Non-standard channel residues are indicated with an *asterisk*. Many Archaeal A-family oxidases are missing important K-channel residues

Family	A	A	A	A	A	A	A	A	A	B	B	B	D	E	F	G	H	sNOR	eNOR
Subfamily	IV	XII	XIII	XIV	XV	XVI	XVII	XVIII	UNC	III	IV	V							
PDB	1M56									1XME									
K-channel	Y288	Y	Y	Y	Y	Y	Y	Y	Y	Y237	Y	Y	Y	Y	Y	Y	Y	N*	Q*
	T359	–	S	S	S	T	S	–	T	S309	S	S	S/T	S	–	S	S	–	S
	K362	–	K	K	K	–	H*	–	K	T312	T	T	T	T	–	T	T	–	H*
	S299	–	S	–	S	–	Y*	–	S	Y248	Y	Y	Y	Y	–	Y	Y	Y	Y
	E101	–	E	E	E	–	E	–	E	E15	E	E	E	E	–	E	E	–	E
D-channel	E286	E	E	E	E	E	E	E	E										
	Y33	Y	Y	Y	Y	Y	Y	Y	Y										
	N139	N	N	N	N	N	N	N	N										
	N121	N	N	N	N	–	N	N	N										
	N207	N	N	N	N	E*	N	N	N										
	D132	D	D	D	D	D	D	D	D										

UNC Unclassified subfamily

* Non-standard amino acid substitutions

Fig. 2 Sequence alignment of representatives from each Archaeal heme–copper family. ► Residues important for channel formation are indicated below the alignment. Sequence identifiers are given in Table 3. Sequences for known crystal structures are also included for reference. (*Rhodobacter sphaeroides* [A1200559a] is an A-family oxidase, while *Thermus thermophilus* [B1110763] is a B-family oxidase. Both species are members of the Bacteria domain.) For space considerations non-homologous regions have been removed from the alignment

from the A-type oxygen reductases (Soulimane et al. 2000). However, subunit IIa is not homologous to the second helix. The B-family of oxygen reductases can be subdivided into five subfamilies (I, II, III, IV, and V) based on groupings with greater than 50% sequence identity in the core subunit. Subfamilies IV and V are found in Archaea exclusively (Table 4). Archaeal members of the B-family have been studied in *Aeropyrum pernix* (Ishikawa et al. 2002), *Pyrobaculum oguniense* (Nunoura et al. 2003), and *Natronobacterium pharaonis* (Mattar and Engelhard 1997).

Mapping of conserved residues (Fig. 2) onto the crystal structure for the *Thermus thermophilus* ba_3 oxidase shows that there is only one conserved proton input channel, a modified K-channel analog (Fig. 1B). The residues which were previously identified to form an analogous D-channel and an alternative proton channel (Q-channel) (Soulimane et al. 2000) are not conserved across the family. The pattern of conserved residues, therefore, suggests that the B-type oxygen reductases have only one proton channel, analogous to the K-channel in the A-family oxygen reductases. This is being tested experimentally.

5.3 C-Family

The C-family of oxygen reductases, also known as the cbb_3 oxidases, is the second largest oxygen reductase family (24%) after the A-family (71%) found in genomic and community DNA sequences. There are no C-family oxygen reductases found in the Archaea in the sequences collected to date. It is noted that two subunits in the C-family oxygen reductases contain heme c. The ability to synthesize c-type cytochromes is unusual and is sporadically distributed through the Archaea (Bertini et al. 2007). Hence, most Archaea do not have the machinery needed to correctly assemble the proteins in the C-family of oxygen reductases.

5.4 Families D Through H

The classification scheme introduced by Pereira et al. (2001) and colleagues would combine the enzymes represented in the D-, E-, F-, G-, and H-families

```
                  --Helix XIII-----  ----------Helix IX----------  ----------Helix X----------  ----------Helix XI----------  --
A1200559a         VIAVPT-GIKIFSWIATM---TPMLWALGF-LFLFTV-GGVTGIV-LSQASVDRYYHDTYYVVAHFHYVMSLGAVFGIFAGIYVFLGKM---WAGKLHFWWMFVGANLTFFPQHFLGRQ-GMPRR---TW
A5010020          LISIPF-EMAVMSFIFTLL---VPMLFAVCA-LLNFII--GGSTCVY-LGSIAIDRGFRGTYWVVAHFHYILVGTVTLGLIAGLYYWWPKI---RLGKIHFALLMLGVNLTFLPQFALMDM---PRR---PL
A5010160          AAVPAS-AMIVFNFVATM---SPMMWSIGG-IALFFS-AGAGCVL-NAAMPIDFTTHDTYWVVGHFHLFVMGTIAFGSIGFFYYWFPFV---KLGKIHFVFSFVGAVLLFFTQHILGIY-GMPRR---AM
A5010718          AIAIPS-GVKVLWTATL---TPTILLISF-IVMFLL-GGITGVF-FPLVPIDYALNGTYFVVGHFHYWVYA-ILVALLGALFYYFPFW---DLGKTGAILLVAGTFLTATGMSIAGIL-GMPRR---PF
A501X004          AVAIPS-GVKVFWWVATL---APMLFMLTF-LLFFVV-GGVTCVF-FPVVTLDLHLQDTYFVLGHFHYIV-NAIAFGALGALLYYFPHL---RLAVLSWGLITVVGGFMAYTLMSAAGIL-GSPRR---PY
A5020319b         GISLPF-DLMVFSLIYTM---TPFLFSLGA-LILFII-GGITGVF-LGAIVLDYQFRGTYWVVAHFHYVMVAGAT-ALFGGLYYWYPKI---TLGKIQFGVYFLGFNLLIYFPMFTAWET---PRR---IW
A5020320          AISIPS-AVKVFNWITTM---APMLFCIGF-VQNFII--GGVTCVF-LAVIPIDLILHDTYYVVGHFHYIVGAIGFALFAASYYWFPMV---RLAHAHFWTALVCSNATFLAMLWLGYG-GMPRR---TA
A5020496          TISIPT-SMLIVGLIATM---TPILFALAA-STSFMI-GGVSGVT-QSSLAIDTIVHGDYYYVAHFHYFMVGVAVMGLMGNLYYYFPKF---KLGRIHWAFAYPGVNILYFPMFFLYDL---PRR---IF
AAACY01004662     LVAVPT-GIKIFNWLMTM---THTLWALGF-LVTFTL-GGISGMF-FPSIAMDTHLHESYFVVAHFHYVLVGGTVFGFFAGIYYWWPKA---KLGYLHFLTAFVSYNALFWFMHHRLGVW-GMTRR---GW
B1110763          FVAVPS-LMTAFTVAASL---NPAFVAPVLGLLGFIP-GGAGGIV-NASFTLDYVVHNTAWVPGHFHLQVASLVTLTAMGSLYWLLPNL---RLGLAVVWLWFLGMNIMAVGLHWAGLL-NVPRR---VP
B501X004          LVAVPS-FTTAFNVIATL---DPVFTGITFAILLFGI-AGFSCVV-NASFNVNYNVHNTAWIVGHFHLFVGGGVTLTFIATSFLLVPLL---KFLIAVPTLWFVSQLIFGISYHIVGLL-HAPRR---PW
B5020320          MLLLPS-LLTFAFTVVASL---KPSFSAMALAGLVFAA-GGFSGMI-NAGMNINYLLIHNTIWVPGHFHLFVGTAFALTMMGVAYWLVPQI---TLAAIQPFVMVIGMTLMSNAMHRGGLA-GLPRR---EM
D501X001          AVVVPS-MMTFLNIWATA---NVITAFVATSFAGAIA-AGVTGIA-NATIAFDSIVHNSMWVVGHFHAMILLSIVPAAMAVLYFMIPMM---KMAWIHYIGYTIGASILIIGFEMIGFY-GVVRR---FA
E5010718          ILATGS-GLTVLNLGLTI---DPVGMGALISLIGFIL-AGAQALV-LPENSINPLFHNSYYVVGHFHLMIWTLIIMGYTTVFLDMLRTS---KWMPIGMIWFTAFPMGVGYAMSVAGYL-GFLRR---PY
F5010720          ALIAIP-FAAFWLLFFIT---DTGLAFLYAAAIWNII-GGIQAEPTNPVQAVDFTIHNTAWIFSHFHIMLALYSVGGVFGAVFAAGPYM---KLSWLHFWGWQACMGLFVTASAIGGFY-GLIRR---PY
G5010896          GISIVS-AVSVFALLAMM---PPYMFSLIA-MFAWLE-AGYSGVA-QAIVAWNLYLHNTLWVVAHFHTMALLNIAMVAIALIYYLVPKA---KWSKVHFWMTLVGGFGFVNTWFAEGVL-GVPRR---NL
H5020905          LVVIPS-ATTIFNYIASL---TPMLFVVNG-IFDFII--GGITGVM-QSMLVVNIQVHGTYWVTGHFHFIFMGITTCILFASYVMLWPTI---RLATWHMALTAIGSFIMSMGWTIGGFL-GMPRY---VY
sNOR1070753       AVGVPAFFVTIVGSLGLL---APVIFILAG-LWGWTV-GGMAAVI-DGTIAINNVMHNTLWVPAHFHSYYLFGAAGVTTAYLFHRLSGV---ALARRLAWLYAIGAGGFVLAFFLSGLQ-SVPRR---GL
eNOR5020447       GAAFAS-MIHAFAIPAGL---NPVFSATIFSIILFGFMGGITGVL-MGQLQLNMTWHNTFATVGHFHGTVALGTTLAFMGLVFFVIRTM---KLASLVPYFYAGAMGIAVLVMMMVVGILKYGVPRR---PL
K-channel                    * *
D-channel
Heme ligands                                                                  * *

                  ----------Helix XII-----
A1200559a         NFVSSLGAFLSFASFFFLGVTFY
A5010020          NQLSTLGAFIFGGSMAIGLVNFLY
A5010160          NQIATVGAMLIGVGMAIFLGNMIH
A5010718          QFMASVGAVLTGIGLFILAGVLVH
A501X004          HVGMTIFTIILAAGLVIYFANLLY
A5020319b         HSMATVGGFILGASFLIMFVNLFV
A5020320          HRLATVGAFLIGVSTLIWLFNMAT
A5020496          NEIATAGAFIFGLSFLIMFANFAW
AAACY01004662     NMFITVSGFIFFFSNFIMVFNMIV
B1110763          MVFNVLAGIVLLVALLLFIYGLFS
B501X004          LQLGAIGGVIFALGGALFLLLTFA
B5020320          QLQIAIGGTLLFVSLALFLLVMVG
D501X001          ENLATAGALIAEIATLVWFVNLVA
E5010718          NLVFSFLAEIGIPGLLLTLFVGMF
F5010720          YQLIMIGGWVAGFSLIVFTFNLIL
G5010896          DLIGVIFAIILGIAQGIFAVKLFN
H5020905          QDIAIAGGVIIGIGQLFFLANLIK
sNOR1070753       AQAAVPFVLLLVIAFGGLLLMALR
eNOR5020447       FLIIGPAAVLAAIIAGALFVVVAVL
K-channel
D-channel
Heme ligands
```

with those in the B-family. We have separated these into distinct families based on the extreme divergence that is evidenced by their sequences. These five families are unique to Archaea, and some are represented by only a single sequence entry. As additional sequences become available, it is likely that the number of highly divergent oxygen reductases will increase substantially.

D-Family

The family of oxygen reductases related to *DoxB* from *Acidianus ambivalens* (Purschke et al. 1997) has been reclassified as the D-family. The enzyme was originally isolated from *Acidianus ambivalens* as a complex of five proteins encoded by two operons, DoxBCE and DoxDA. Recently, it has been shown that these two operons in fact encode two separate complexes; a thiosulfate:caldariella quinone oxidoreductase (DoxDA) and a caldariella quinol:oxygen reductase (DoxBCE) (Muller et al. 2004). The complexes together couple the oxidation of thiosufate to the reduction of O_2.

Subunit II of the oxygen reductase complex is very divergent and does not contain residues required for the formation of the Cu_A site. This is not surprising since the complex oxidizes caldariella quinol and other oxygen reductases which oxidize quinols are, as a rule, also missing the residues which bind Cu_A. It has been suggested that a caldariella quinone may be permanently bound to subunit I as a redox active cofactor, functionally taking the place of the Cu_A site. This cofactor would be capable of sequentially transferring two electrons to the low spin heme a (Schafer 2004).

Structural modelling and sequence analysis of subunit I have identified the presence of a modified K-channel, identical to the one found in the B-family (Tables 1 and 4 and Figs. 1B and 2). No other conserved channels can be defined based on the pattern of residue conservation. It was previously suggested that the D-family has a D-channel analog, with E80 functionally replacing the glutamate (E286 in Rhodobacter) at the top of the D-channel in the A-family (Gomes et al. 2001). However, this glutamate (E80) is not conserved in any of the other D-family members. This observation, combined with the fact that most of the other residues implicated in the formation of D- and Q-channel analogs (Victor et al. 2004) are not conserved, strongly suggests that the D-family only has one functional proton input channel.

E-Family

The E-family members are related to the *SoxB* oxygen reductase from *Sulfolobus acidocaldarius* (Lübben et al. 1992). The proteins encoded by the SoxABCD operon form a supercomplex with SoxLN which is an Archaeal equivalent of a bc_1 complex. It appears that caldariella quinone is the ultimate electron donor, with the electrons being passed from the quinone to the SoxLN complex, which transfers them directly to the SoxABCD complex (Schafer 2004). This is similar to the situation with the SoxM supercomplex (also found in *Sulfolobus acidocaldarius*), except in this case the oxygen

reductase component (*SoxB*) is an E-family oxygen reductase. Structural analysis shows that the E-family contains a conserved K-channel similar to the one found in the B-family (Tables 1 and 4 and Figs. 1B and 2).

F-Family

The oxygen reductase genes related to *FoxA* from *Sulfolobus metallicus* (Bathe and Norris 2007) have been classified as the F-family. These genes form part of a gene cluster that is highly expressed when the cells are grown on ferrous iron or pyrite but not sulfur. This implies that this oxygen reductase family is part of a respiratory chain that specifically couples the oxidation of ferrous iron [Fe(II) oxidized to Fe(III)] to the reduction of O_2.

Structurally, the F-family is a two subunit complex with subunit II containing a Cu_A binding domain. Analysis of subunit I shows that it does not have any conserved proton input channels (Tables 1 and 4 and Figs. 1B and 2). Unless non-conserved residues comprise the proton delivery channel, this suggests that the F-family of oxygen reductases cannot be electrogenic since both the electrons and protons would have to be derived from the same side of the membrane, and no proton channels are available for proton pumping. This situation is similar to that of the NO reductases. The putative lack of proton pumping may not be surprising, considering the driving force available from Fe(II) oxidation and the acidic environment in which these organisms grow. However, one might expect at the least that protons would be utilized from the cytoplasm to maintain a neutral internal pH. This is another example in which biochemical studies are needed to test the validity of predictions inferred by sequence analysis.

Interestingly, the acidophilic bacteria *Thiobacillus ferrooxidans* contains a modified A-type oxygen reductase that is expressed when grown on ferrous iron (Yarzabal et al. 2004). This modified A-type oxygen reductase is also missing the residues which form the K-channel, though the D-channel appears to be present (Ingledew 2004). Possibly, this bacterial A-type oxidase also lacks the ability to pump protons but, in this case, protons are utilized from the cytoplasm to form water at the enzyme active site. This would be an example of the same evolutionary solution (lack of proton channels) to an environmental problem (low pH) being implemented in two different families.

G- and H-Families

Members of the G- and H-families are recent discoveries and, so far, represented by only one sequence entry for each. Neither family has been biochemically or genetically characterized. The G-family sequence was identified in the Crenarchaeota *Caldivirga maquilingensis (IC-167)* in an operon encoding homologs to subunits I and II along with genes for sulfocyanin, polyferredoxin, and *SoxL* and *SoxC* homologs. These proteins could form a supercomplex similar to the one found in the E-family. The H-family was identified in *Thermoplasmatales archaeon (Gpl)* assembled from acid mine drainage

(Tyson et al. 2004). It is located within an operon encoding subunit I and II homologs along with a CtaB homolog. *Caldivirga maquilingensis (IC-167)* and *Thermoplasmatales archaeon (Gpl)* do not appear to encode any other heme–copper oxygen reductases. This implies that both of the enzymes representing these families are functional in their respective organisms for oxygen respiration.

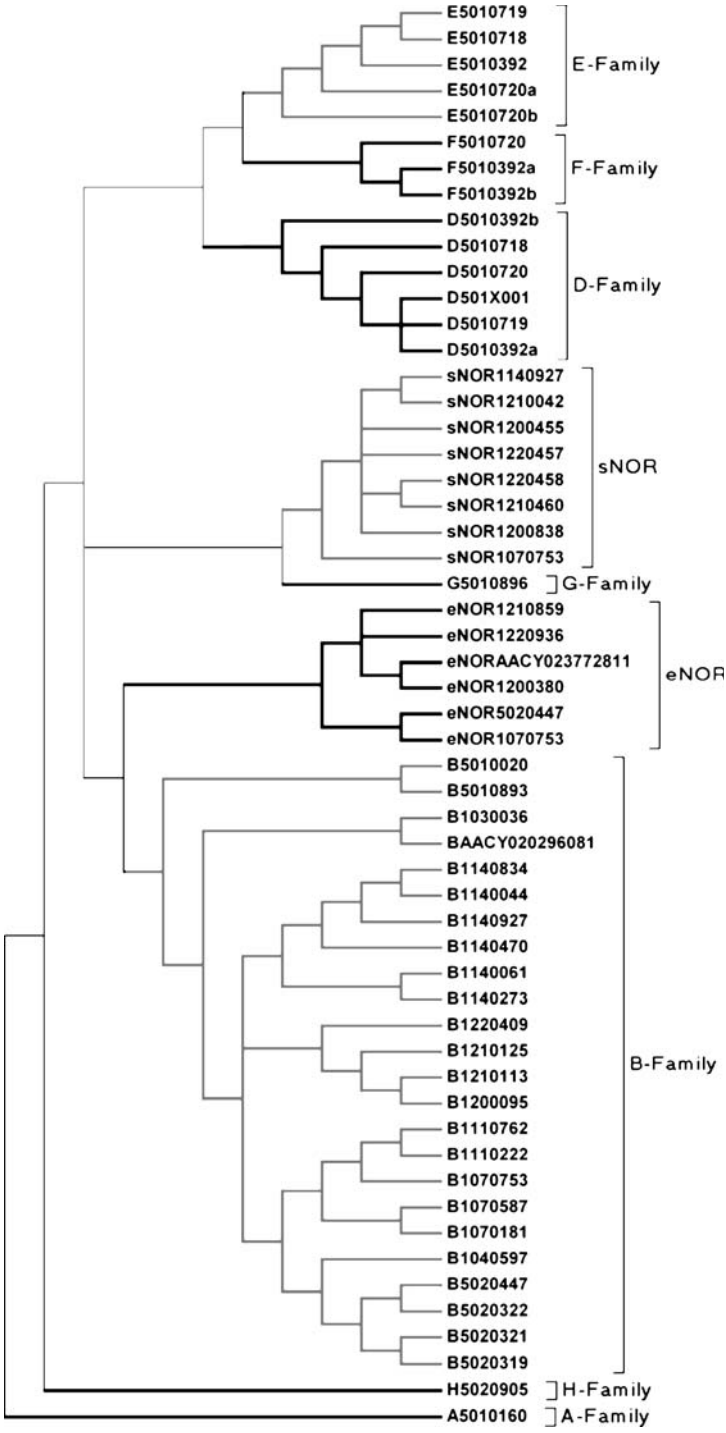
In these two enzymes, subunit II has the residues required for the formation of a Cu_A binding site and subunit I has the residues needed for the formation of a modified K-channel, analogous to the one found in the B-family of oxygen reductases (Tables 1 and 4 and Figs. 1B and 2). Genomic and community sequencing projects in progress along with biochemical analyses will be required to further elucidate the properties of these new families.

6 Distribution and Evolution of the Heme–Copper Superfamily

The phylogenetic and family distribution of heme–copper superfamily members found within sequenced Archaeal genomes is given in Tables 1 and 2. The Archaea encode members from seven of the eight currently identified heme–copper oxygen reductase families, whereas the Bacteria only have members from three; the A-, B-, and C-families. The D-, E-, and F-families have so far only been found in the Sulfolobales class of the Crenarchaeota. Figure 3 shows the phylogenetic positions of the newly identified heme–copper families. Phylogenetic analyses strongly suggest that the heme–copper oxygen reductases originated within the Bacteria and members were later transferred to the Archaea. This leads to the question: Why is there so much sequence divergence within Archaeal members of the superfamily after they have acquired them from Bacteria? One answer may be that Archaeal membranes select for different sequence characteristics than do Bacterial membranes, leading to rapid divergence. Another might be that since most of the oxygen reductases in Archaea have only one channel there is less selection pressure on most of the protein, and hence a higher rate of evolution.

The nitric oxide reductase members of the superfamily which are found in Archaea are predominantly from the qNOR family. Very few Archaea utilize cytochrome c as an electron carrier, which would exclude the use of the cytochrome c-requiring cNOR family. The qNOR family has a very sporadic and phylogenetically discordant distribution with members found in both the Crenarchaeota and Euryarchaeota. Phylogenetic analysis of the qNOR family

Fig. 3 Phylogenetic analysis of new Archaeal heme–copper families. Sequence identifiers are given in Table 3. Identifiers starting with a 1 represent Bacteria sequences ►


E5010719
E5010718
E5010392
E5010720a
E5010720b
E-Family
F5010720
F5010392a
F5010392b
F-Family
D5010392b
D5010718
D5010720
D501X001
D5010719
D5010392a
D-Family
sNOR1140927
sNOR1210042
sNOR1200455
sNOR1220457
sNOR1220458
sNOR1210460
sNOR1200838
sNOR1070753
sNOR
G5010896
G-Family
eNOR1210859
eNOR1220936
eNORAACY023772811
eNOR1200380
eNOR5020447
eNOR1070753
eNOR
B5010020
B5010893
B1030036
BAACY020296081
B1140834
B1140044
B1140927
B1140470
B1140061
B1140273
B1220409
B1210125
B1210113
B1200095
B1110762
B1110222
B1070753
B1070587
B1070181
B1040597
B5020447
B5020322
B5020321
B5020319
B-Family
H5020905
H-Family
A5010160
A-Family

also shows that it is very likely that the Archaea acquired them via lateral gene transfer from Bacteria.[1]

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