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Biochimica et Biophysica Acta 1505 (2001) 185–208

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Review

A novel scenario for the evolution of haem–copper oxygen reductases

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Received 14 November 2000; received in revised form 14 February 2001; accepted 15 February 2001

Abstract

The increasing sequence information on oxygen reductases of the haem–copper superfamily, together with the available three-dimensional structures, allows a clear identification of their common, functionally important features. Taking into consideration both the overall amino acid sequences of the core subunits and key residues involved in proton transfer, a novel hypothesis for the molecular evolution of these enzymes is proposed. Three main families of oxygen reductases are identified on the basis of common features of the core subunits, constituting three lines of evolution: (i) type A (mitochondrial-like oxidases), (ii) type B (ba_3-like oxidases) and (iii) type C (cbb_3-type oxidases). The first group can be further divided into two subfamilies, according to the helix VI residues at the hydrophobic end of one of the proton pathways (the so-called D-channel): (i) type A1, comprising the enzymes with a glutamate residue in the motif –XGHPEV–, and (ii) type A2, enzymes having instead a tyrosine and a serine in the alternative motif –YSHPXV–. This second subfamily of oxidases is shown to be ancestor to the one containing the glutamate residue, which in the Bacteria domain is only present in oxidases from Gram-positive or purple bacteria. It is further proposed that the Archaea domain acquired terminal oxidases by gene transfer from the Gram-positive bacteria, implying that these enzymes were not present in the last common ancestor before the divergence between Archaea and Bacteria. In fact, most oxidases from archaea have a higher amino acid sequence identity and similarity with those from bacteria, mainly from the Gram-positive group, than with oxidases from other archaea. Finally, a possible relation between the dihaemic subunit (FixP) of the cbb_3 oxidases and subunit II of caa_3 oxidases is discussed. As the families of haem–copper oxidases can also be identified by their subunit II, a parallel evolution of subunits I and II is suggested.

Keywords: Haem–copper oxidase; Evolution; Oxygen

1. Introduction

Terminal oxidases of membrane-bound electron transfer chains from aerobic organisms catalyse the reduction of dioxygen to water, coupling the redox energy to proton translocation through the cytoplasmic (or mitochondrial) membrane [1–5]. Most terminal oxidases belong to the haem–copper superfamily [6,7]. This superfamily is quite diverse in terms of electron donors, subunit composition and haem types. While the mitochondrial enzyme contains up to 13 subunits, the prokaryotic ones have a much lower number of subunits (generally four); however, the 3D structures of the bovine [8,9] and the bacterium *Paracoccus denitrificans* [10,11] enzymes show that its subunits I and II are remarkably similar. The superfamily is defined by the presence, in subunit I, of a six-coordinated, low-spin haem and a

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0005-2728/01/$ – see front matter
PII: S0005-2728(01)00169-4

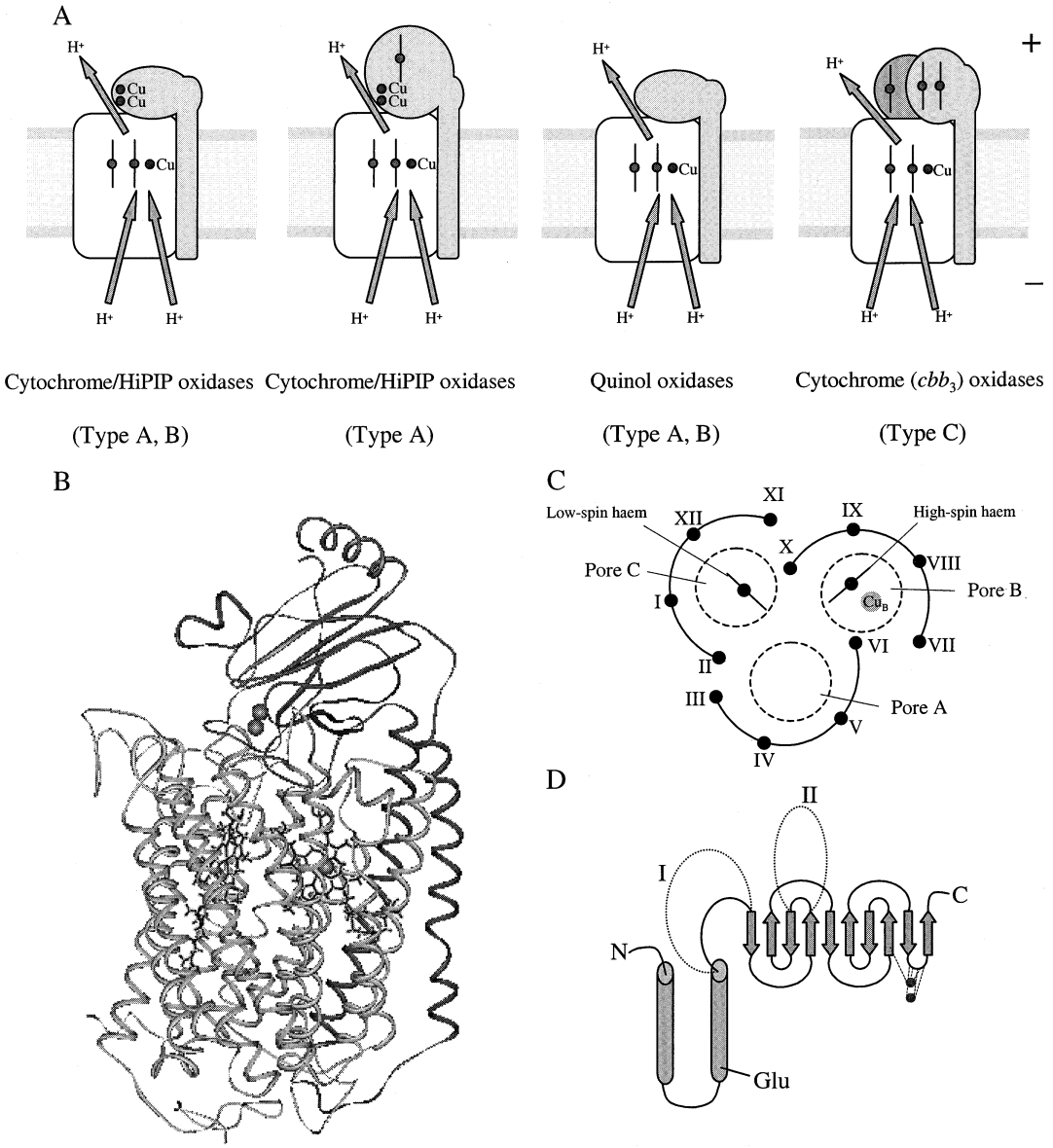

Fig. 1. (A) Schematic representation of the main types of terminal oxidases. Proton pathways are schematically represented by arrows. (B) Crystal structure of subunits I (light grey) and II (dark grey) from *P. denitrificans* aa_3 oxidase (PDB code 1AR1). (C) Schematic view of subunit I of the same enzyme observed from the negative side of the membrane (adapted from [10]). (D) Schematic representation of subunit II structure for type A or B oxidases, including the binding sites for Cu_A in cytochrome/HiPIP oxidases. The dashed loops represent the different insertions observed in type A1 (loop II) and type A2 (loop I) or type B (loop I) oxidases. The conserved glutamate residue (GluII-78), which is the possible entry of the K-channel, is marked.

unique bimetallic centre (which gives this family its name) composed of a high-spin haem and a copper ion, Cu_B (Fig. 1). The low-spin haem is the immediate electron donor to the binuclear centre, where the catalytic reaction takes place. A broad subdivision of this superfamily was previously established, on the basis of amino acid sequence alignments of subunit I: (i) the Cox type, including the enzymes more similar to the mitochondrial cytochrome oxidase, and (ii) a highly divergent group, the FixN type, also named cbb_3 oxidases [6,7,12]. The Cox-type oxidases have a second subunit, with one or two transmembrane helices and a peripheral domain: in cytochrome and HiPIP (high potential iron–sulfur protein) oxidases this domain contains a mixed-valence binuclear copper site (Cu_A), while in quinol oxidases

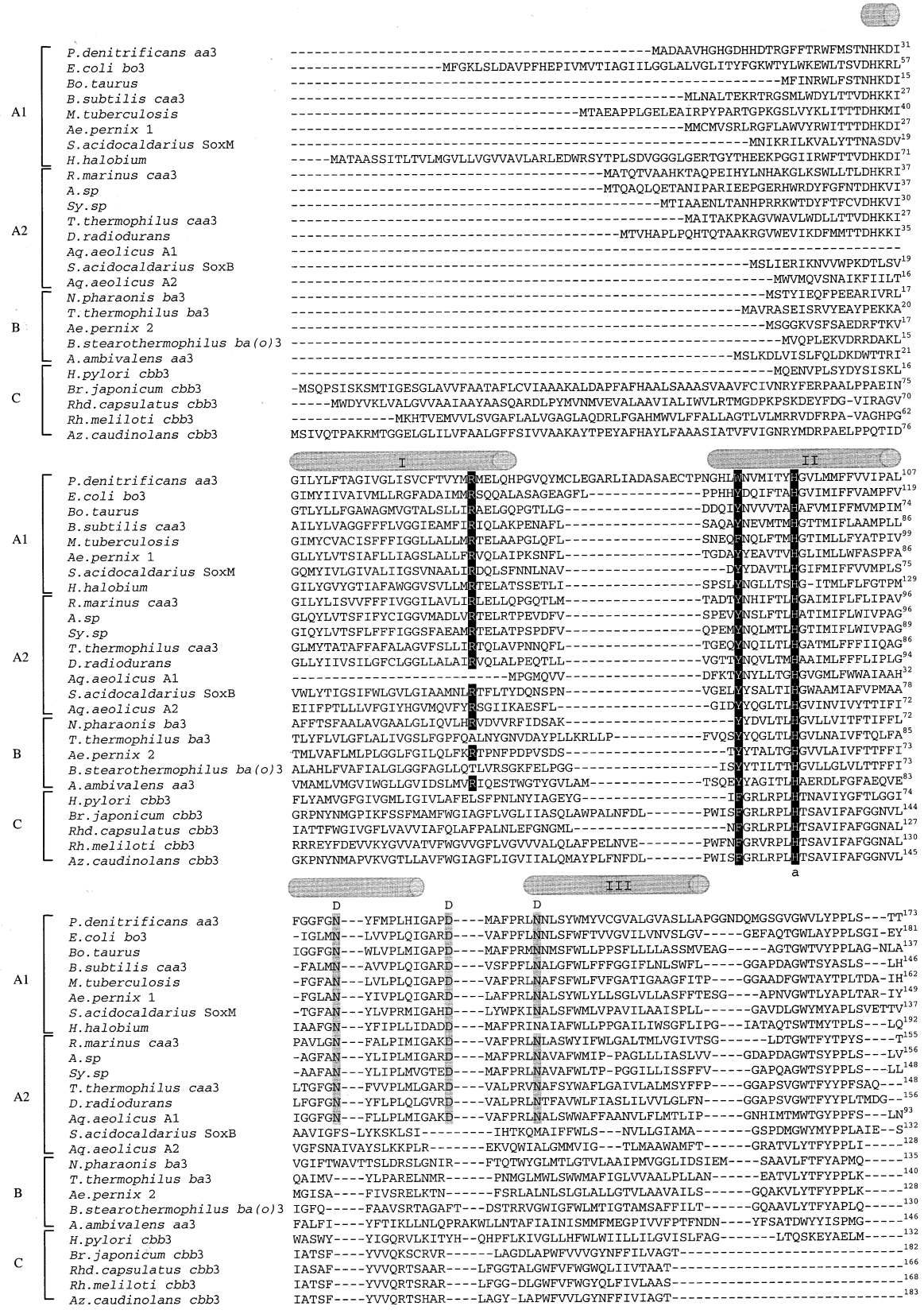
A1
A2
B
C
P.denitrificans aa3
E.coli bo3
Bo.taurus
B.subtilis caa3
M.tuberculosis
Ae.pernix 1
S.acidocaldarius SoxM
H.halobium
R.marinus caa3
A.sp
Sy.sp
T.thermophilus caa3
D.radiodurans
Aq.aeolicus A1
S.acidocaldarius SoxB
Aq.aeolicus A2
N.pharaonis ba3
T.thermophilus ba3
Ae.pernix 2
B.stearothermophilus ba(o)3
A.ambivalens aa3
H.pylori cbb3
Br.japonicum cbb3
Rhd.capsulatus cbb3
Rh.meliloti cbb3
Az.caudinolans cbb3
I
II
III
D
a

Fig. 2.

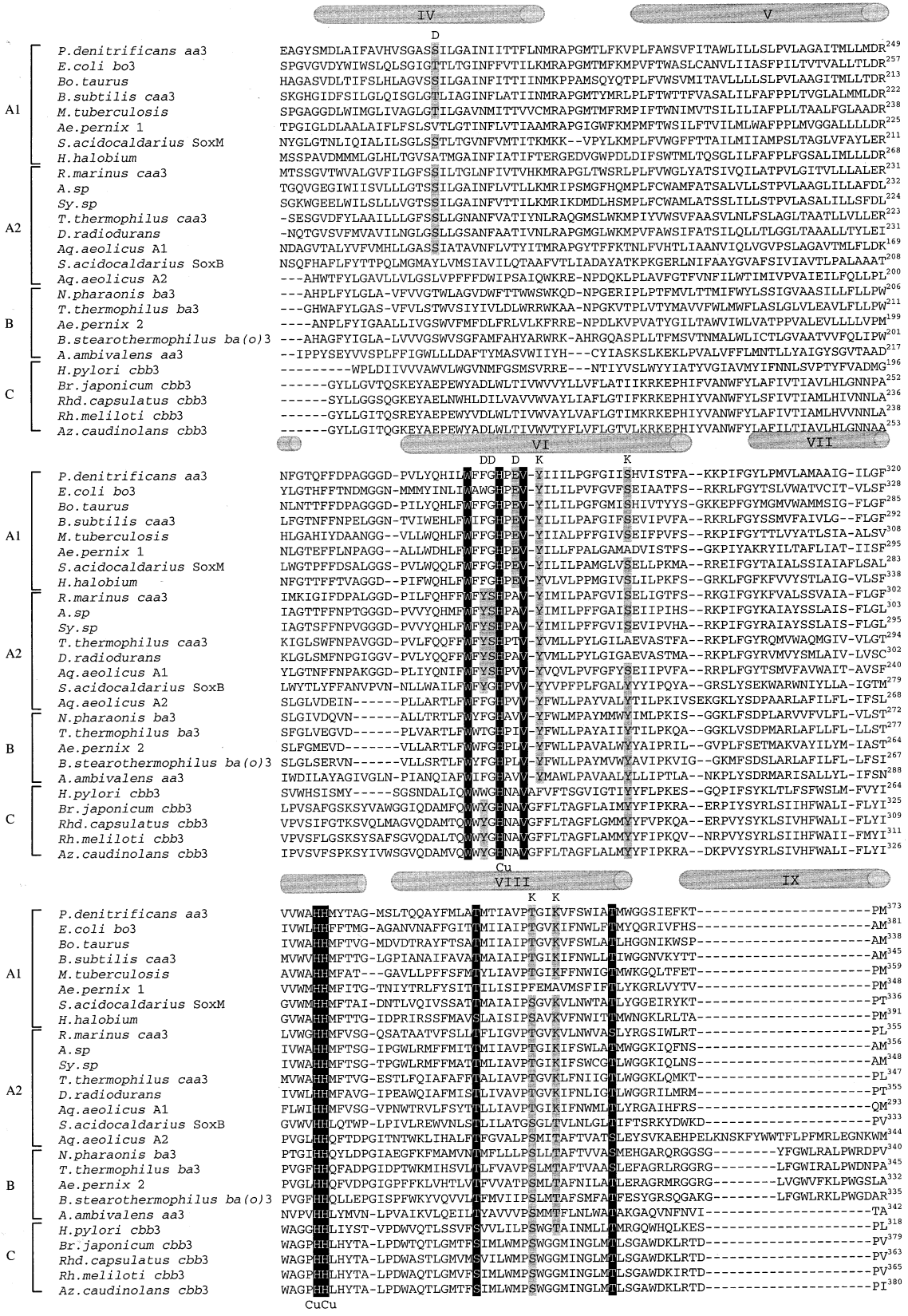

Fig. 2 (*continued*).

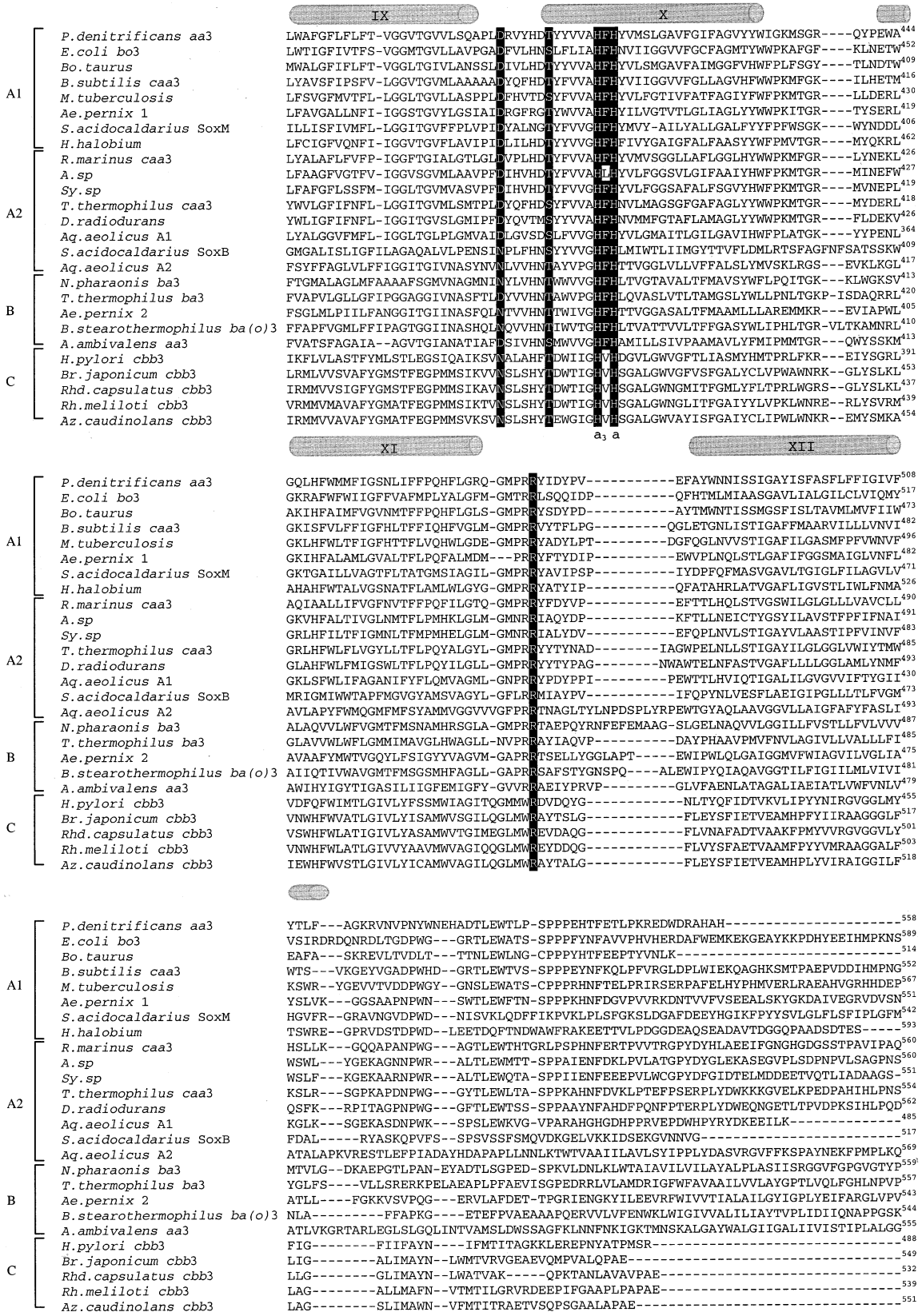

Fig. 2 (*continued*).

Fig. 2. Amino acid sequence alignment of haem–copper oxidase subunit I (accession numbers in parentheses). A, B and C refer to the oxidase families as described in the text. *P., Paracoccus* (P08305), *E., Escherichia* (P18400), *Bo., Bos* (P00396), *B., Bacillus* (*subtilis* caa_3, P24010, *stearothermophilus* ba_3, O82837), *M., Mycobacterium* (O53290), *Ae., Aeropyrum* (1, Q9YDX6, 2, BAA80883), *S., Sulfolobus* (SoxM, P39481, SoxB, S21042), *R., Rhodothermus* (CACO8532), *A., Anabaena* (*Anabaena* sp. strain PCC 7937, Z98264), *Sy., Synechocystis* (*Synechocystis* sp. strain PCC 6803, Q06473/P73261), *T., Thermus* (caa_3, P98005, ba_3, CAB06339), *D., Deinococcus* (G75251), *Aq., Aquifex* (A1, C70488, A2, E70488), *N., Natronobacterium* (CAA71525), *A., Acidianus* (CAA69980), *H., Helicobacter* (AAD0571), *Br., Bradyrhizobium* (Q03073), *Rhd., Rhodobacter* (AAC46108), *Rh., Rhizobium* (S39988), *Az., Azorhizobium* (CAA52429). Strictly and almost strictly conserved amino acid residues are shaded in black. Amino acid residues of the proton channels are shaded in grey and labelled D (D-channel) and K (K-channel). The histidine ligands to the low- and high-spin haems and to Cu_B are indicated by a, a_3 and Cu, respectively. Transmembrane helices for the *P. denitrificans* aa_3 oxidase are indicated by cylinders on top of the alignment. Alignments were made using Clustal W Version 1.6 [90] and manually adjusted.
←

this centre is absent. The cbb_3 oxidases contain two other subunits, with one and two *c*-type haems (Fig. 1).

Phylogenetic relations among the haem–copper oxidases were established by amino acid sequence analysis [13,14] showing that cbb_3 oxidases were the most distant members of the haem–copper superfamily. It was also suggested that these oxidases were closer to a putative primordial cytochrome oxidase from which the extant enzymes have evolved. Quinol oxidases were proposed to evolve from cytochrome oxidases by loss of the Cu_A centre, as this loss could be easily reverted by mutating a few residues, as it was done for the *Escherichia coli* bo_3 quinol oxidase [15]. Musser and Chan [16] presented a different proposal for the evolution of the oxidases, arguing that sequence analyses are only valid if functional homology is observed. These authors claimed that in cytochrome *c* and quinol oxidases proton translocation is not achieved by the same mechanism: for cytochrome *c* oxidases they proposed a proton pumping mechanism in which Cu_A would be involved, while for quinol oxidases a Q-cycle should be operative.

Neither of the above evolutionary hypotheses considered the presence of proton channels inside the protein enabling the access of protons to the catalytic centre, which were suggested by sequence analyses, site-directed mutagenesis studies and crystallographic structures [2,8–11,17–21].

In this article, features common to all known members of the haem–copper oxidase superfamily are identified. Taking into account the increasing number of full sequences available and considering specific amino acid residues involved not only in the binding of the prosthetic groups, but also making part of the different proton channels, a classification for oxidases is proposed. Based on the same approach, the evolutionary relations among the members of the haem–copper superfamily are discussed. A new proposal is raised on the basis of what is common, functionally relevant to all haem–copper oxidases in sequence and mechanistic terms, which correlates quite well with the 16S rRNA-based organismal phylogeny. The paper is divided into three main parts: first, the most important features of subunits I and II are presented, which allow the establishment of a structural and functional classification of the oxidase families; secondly, the distribution of these families among the prokaryotic domains is analysed and a new hypothesis for the molecular evolution of these enzymes is proposed; finally, a possible parallel evolution of subunits I and II is discussed.

2. Subunit I

Only subunit I is common to all haem–copper oxidases, which stems from the fact that it contains the catalytic centre, the haem–copper binuclear site, and its immediate electron donor, the low-spin haem (Fig. 1). Hence, the residues binding these prosthetic groups (six histidines: HisI-94, HisI-276, HisI-325, HisI-326, HisI-411 and HisI-413, *P. denitrificans* numbering[1]) are strictly conserved in all haem–copper oxidases (Fig. 2). The prokaryotic oxidases may contain quite diverse haems: A, B, O and derivatives

[1] Throughout the text, and unless otherwise stated, the amino acid numbering of *P. denitrificans* cytochrome aa_3 is used, as in the PDB file code 1AR1.

Table 1
Conserved residues in haem–copper oxidase subunit I, besides the metal ligands

Residue	Function
Valine (ValI-279)	O_2-channel
Tryptophan (TrpI-272)	π interaction with HisI-326
Arginine (ArgI-474)	H-bond to δ-propionate of the low-spin haem
Arginine (ArgI-54)[a]	H-bond to formyl group of haem *a*
Aromatic (TrpI-87)	H-bond to one of the propionates of the low-spin haem
Threonine/serine (ThrI-344)	H-bond to HisI-325
Aspartate/asparagine (AspI-399)	H-bond to one of the propionates of the high-spin haem
Phenylalanine (PheI-412)[b]	Possible electron transfer between the haems
Threonine/serine (ThrI-361)	Unknown
Threonine/serine (ThrI-405)	Unknown

[a]Only in haem *a* low spin-containing oxidases.
[b]Absent in cbb_3 oxidases and in the oxidase from *Anabaena* sp.

of the A and O haems (e.g., [22]), which have been used to designate the enzymes. However, the type of haem does not correlate with organism phylogeny, neither with the type of electron donor nor, which is most important, with amino acid sequence similarities. Therefore, it is quite inappropriate to classify oxidase families using the types of haem.

Subunits I have at least 12 transmembrane helices, which span the lipid membrane and provide the protein scaffold for the redox centres. In the four structures determined to date, these centres are located at approximately 13 Å and 30 Å from the positive (outer or periplasmic) and negative (inner or cytoplasmic) membrane sides, respectively [8–11,23,24] (Fig. 1B). The metal centres are well embedded in the protein, thus avoiding the release of reactive oxygen species during the catalytic cycle. The 12 helices, viewed from the negative side, are arranged in three symmetry-related semicircles (Fig. 1C) forming three pores blocked by aromatic residues (pore A), and the binuclear centre (pore B). Pore C is blocked by the low-spin haem and its hydroxylethylfarnesyl side chain (haem *a* containing oxidases [9,10]) or hydrophobic residues (in the case of haem *b*-containing oxidases [23,24]). It may be anticipated that the same general spatial arrangement is common for all haem–copper oxidases, as deduced by comparing their primary sequences and by structure prediction analyses.

Besides the histidine ligands to the metal centres, only three amino acids are strictly conserved (Fig. 2, Table 1). These residues have been pointed out to be critical for the key processes of oxygen diffusion and proton pumping and electron transfer. Two of these residues are located in helix VI, quite close to the binuclear centre. One is a valine (ValI-279), which was suggested to be part of an O_2 diffusion channel. This residue has been recently mutated to an isoleucine in the bo_3 oxidase from *E. coli*, and a significant increase in K_M for oxygen was observed, without any change in V_{max} [25]. The authors attributed this observation to a partial block of oxygen diffusion. The other helix VI residue is a tryptophan (TrpI-272), proposed to be important for the so-called histidine cycle mechanism, due to its π interaction with the imidazole side chain of the histidine (HisI-326) involved in this process [26]. The third strictly conserved residue is an arginine (ArgI-474), located in the loop connecting helices XI and XII, facing the positive side of the membrane. This arginine is hydrogen-bonded to the δ-propionate of the low-spin haem, and is important for proton exit [27,28].

A few other residues are almost strictly conserved in all oxidases (Table 1). An arginine (ArgI-54) in helix I, which forms a hydrogen bond between its amine side chain and the formyl group of haem *a*. Not surprising, this residue is not present in oxidases known to incorporate a different type of haem at the 'low-spin site', such as the oxidases ba_3 from *Thermus thermophilus*, $ba(o)_3$ from *B. stearothermophilus* and cbb_3 (Fig. 2). Mutations of this residue in the aa_3 oxidase from *P. denitrificans* led to important alterations in the low-spin haem reduction potential and in its rate of reduction [29]. The other residue is an aromatic amino acid (TrpI-87) in helix II (Fig. 2). This residue also forms a hydrogen bond to one of

Table 2
Proton channels in haem–copper oxidases

Domain	Subdomain/group/order/genus	Oxidase	Channel residues					
			'D'[a]		'K'[b]			
			E/Y	Others	K/T	T/S	S/Y	Y
Bacteria	Purple bacteria	aa_3	E	+	K	T	S	Y
		caa_3	E	+	K	T	S	Y
		bo_3	E	+	K	T	S	Y
		bb_3 (ba_3)	E	+	K	T	S	Y
	Gram-positive	aa_3	E	+	K	T	S	Y
		caa_3	E	+	K	T	S	Y
		ba_3 (*B. stearothermophilus*)	Y[c]	−	T	S	Y	Y
	Cyanobacteria	aa_3	Y	+	K	T	S	Y
	Flexibacter, *Bacteroides* and *Cytophaga* (*R. marinus*)	caa_3	Y	+	K	T	S	Y
	Thermus and *Deinococcus*	caa_3	Y	+	K	T	S	Y
		ba_3	T(?)	−	T	S	Y	Y
	Aquificiales	*Aquifex aeolicus* A1[d]	Y	+	K	T	S	Y
		A2[d]	−	−	T	S	Y	Y
	Purple bacteria and *R. marinus*	cbb_3	Y	−	−	S	Y	−
Archaea	*Crenarchaeota*	SoxB (*S. acidocaldarius*)	Y	−	T	S	Y	Y
		SoxM (*S. acidocaldarius*)	E	+	K	S	S	Y
		Aeropyrum pernix 1[d]	E	+	−	−	−	Y
		Aeropyrum pernix 2[d]	−	−	T	S	Y	Y
		aa_3 (*A. ambivalens*)	−	−	T	S	Y	Y
		Pyrobaculum aerophilum[d]	E	+	K	S	S	Y
	Euryarchaeota	ba_3 (*N. pharaonis*)	Y[c]	−	T	S	Y	Y
		Halobacterium halobium[d]	E	+	K	S	S	Y
Eukarya		aa_3	E	+	K	T	S	Y

[a]GluI-278, TyrI-256 (*R. marinus*); Others: AspI-124, AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134 and SerI-193.
[b]LysI-354, ThrI-351, SerI-291 and TyrI-280. This tyrosine is covalently bound to one of the histidine ligands of Cu_B (HisI-276).
[c]This tyrosine is one residue before in the pattern –YSHPXV–.
[d]Only the gene sequence is known.

the propionates of the low-spin haem. In helix VIII, there is always a hydroxyl group from a threonine (ThrI-344) or a serine, establishing a hydrogen bond with one histidine ligand (HisI-325) of Cu_B. An aspartate equivalent to AspI-399 is in some oxidases substituted for an asparagine and is involved in hydrogen bonding with one of the high-spin haem propionates. The mutation of this aspartate residue to an asparagine in *P. denitrificans* aa_3 oxidase did not change the activity of the enzyme [30]. A phenylalanine (PheI-412) may be involved in electron transfer between the haems [5], but it is not present in the cbb_3 oxidases, or in the oxidase from *Anabaena*. The presence of this residue may not be essential, as the edge to edge distance between the haems is only 4.5 Å [8–11]. In the position of *P. denitrificans* ThrI-361 and ThrI-405 there is always a threonine or a serine residue in all oxidases; however, there are no mutagenesis data on these residues, and from the structural information it is not possible to infer their possible (if any relevant) function.

2.1. Proton channels

The catalytic reaction performed by all terminal oxidases, i.e., the reduction of O_2 to H_2O

$$O_2 + 4e + 4H^+ \Rightarrow 2H_2O$$

involves four protons. As the reaction site is well embedded in the protein, intra-protein channels for proton conduction must be always present in subunit I. Proton conduction is most probably achieved by a Grotthus-type mechanism, in which the proton is transferred stepwise between neighbouring water

molecules, stabilised in the protein cavity by interaction with either the backbone amide groups (as in the gramicidin pore) or hydrophilic side chains from polar groups [31–36]. Protonatable residues may also participate in the process. A wealth of mutagenesis data (e.g., [17,19–21]) led to the identification of two such channels in the mitochondrial-like enzymes, for proton delivery to the chemical reaction and for proton translocation. These proton channels were named D- and K-channel according to those conserved residues. The D-channel, besides AspI-124 (D), is composed of the residues AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134, SerI-193 and GluI-278, and is situated in the lower part of pore A and upper part of pore B. The K-channel is composed of the residues LysI-354 (K), ThrI-351, SerI-291 and TyrI-280 (e.g., [5,17]) (Fig. 2, Table 2), being located in pore B. This channel leads directly to the binuclear site. The tyrosine TyrI-280 is covalently bound to one of the histidine ligands of Cu_B (HisI-276), and is proposed to intervene in the catalytic cycle forming a transient tyrosyl radical [17,37–39]. In both the bovine and *P. denitrificans* enzymes, the D-channel appears to be composed of two parts: a hydrophilic one, starting at the surface of the molecule facing the negative side of the membrane and ending at GluI-278, and a hydrophobic one, leading from the glutamate to the binuclear centre [8–11]. The proton conductivity from the glutamate onwards to that site has to be assured by water molecules [35,40,41]. However, inspection of subunit I amino acid sequences shows that the residues of these channels are far from being common to all oxidases.

The enzymes containing the D- and K-channels form a consistent group in terms of amino acid similarities (Fig. 3, Tables 3 and 4), that we classify as the type A oxidases of the haem–copper superfamily. The caa_3 oxidases from the prokaryotes *Rhodothermus marinus* [42], *T. thermophilus* [43] and *Deinococcus radiodurans* [44], oxidase 1 from *Aquifex aeolicus*

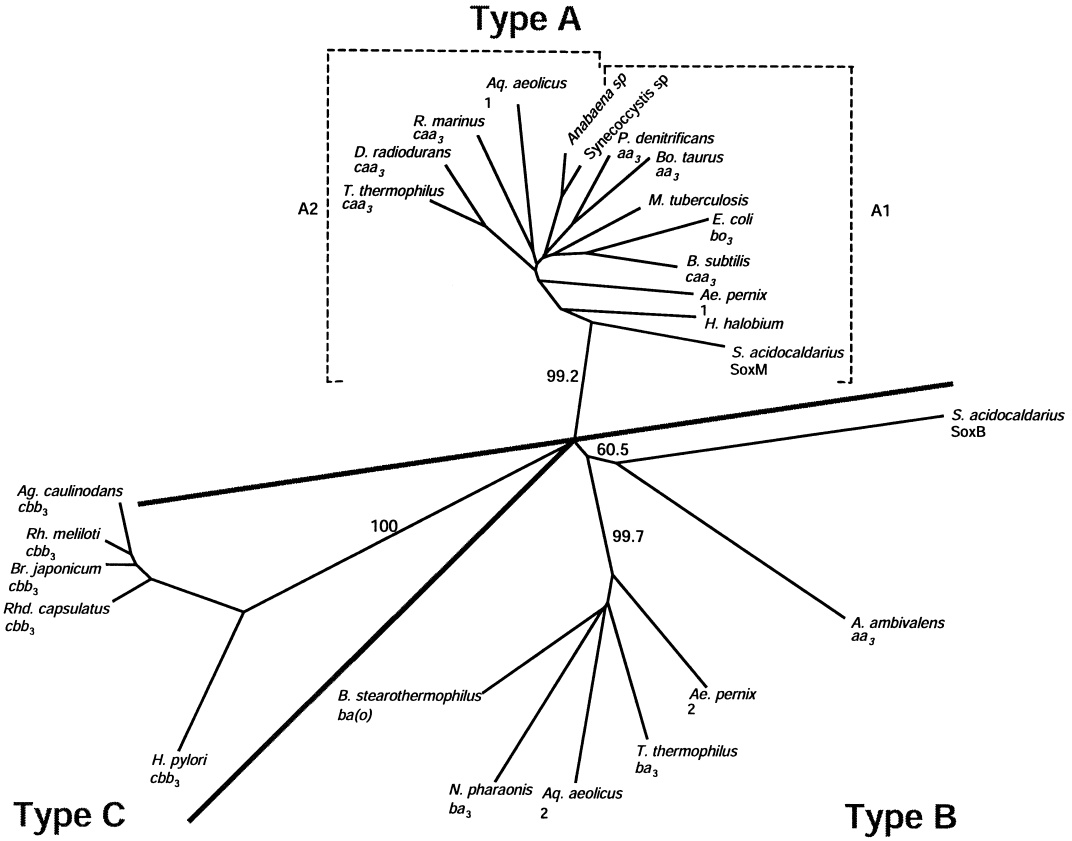

Fig. 3. Phylogenetic tree for the haem–copper oxidase subunit I, based on the amino acid sequence alignment of Fig. 2. The tree was made using Clustal W Version 1.6 [90] excluding positions with gaps and correcting for multiple substitutions. Bootstrap values for the main branches are indicated.

[45], and the oxidases from cyanobacteria [46,47], have all the residues of the D- and K-channels with the exception of the helix VI glutamate (GluI-278) at the hydrophobic end of the D-channel (Fig. 2, Table 2). This residue has been considered a key element for proton transfer, since mutations at this position prevented the enzyme to complete the catalytic cycle and consequently the proton pumping activity [17,19–21]. The *R. marinus* oxidase was shown to reduce completely O_2 to water [42] and later to pump protons [48]. Based on a homology model performed for this oxidase it was shown that the hydroxyl group from a tyrosine residue (equivalent to PheI-274), in helix VI, occupies the spatial place of the carboxyl group of the glutamate [42,49]. This tyrosine residue is conserved in all the above-mentioned oxidases, in a helix VI motif –YSHPXV–, instead of $–X^{274}$GHPE$V^{279}–$ (*P. denitrificans* numbering) (Fig. 2). Furthermore, these enzymes also contain a conserved consecutive serine, which may be relevant for proton transfer. For the oxidases containing the glutamate residue it was proposed, based on theoretical calculations and Fourier transform infrared spectroscopy, that the carboxylate side chain of this residue could adopt two different conformations ('in' and 'out') related by a 180° rotation around the C_β–C_γ bond [41]. The hydroxyl groups of the tyrosine and the serine appear to occupy positions equivalent to the 'in' and 'out' conformations of the glutamate. Hence, it was proposed that the YS motif could be a functional substitute of the glutamate residue [42,48,49]. This was recently corroborated by the observation that a triple mutation in the aa_3 oxidase from *P. denitrificans* (F274Y/G275S/E278A), substituting the glutamate residue by an alanine and introducing the YS motif, results in a fully active enzyme in terms of oxygen reduction and proton translocation [50]. Quite interestingly, the tyrosine residue is also present in other distinct enzymes which lack not only an equivalent to GluI-278, but also other residues of the D-channel, such as *Sulfolobus acidocaldarius* SoxB and the cbb_3 oxidases (see below), suggesting that in these enzymes this tyrosine may be part of alternative proton pathways (Fig. 2, Table 2). These considerations allow the identification of two subfamilies (YS- or E-containing) for the oxidases having the K- and D-channels (Tables 2–4), which are fully supported by the overall amino acid similarities among the members of these subfamilies (Table 4, Figs. 2 and 3). We classify these two subfamilies as type A1 (E-containing) and A2 (YS-containing).

Spread among bacteria and archaea, a second family of oxidases can be defined, as deduced by sequence comparisons (Figs. 2 and 3, Tables 2 and 3), that we designate type B oxidases. In contrast to the known type A enzymes, this subfamily is so far much more diverse. It includes the oxidases 2 from *Aq. aeolicus* [45] and from *Aeropyrum pernix* [51], ba_3 from *T. thermophilus* [52] and *Natronobacterium pharaonis* [53], $ba(o)_3$ from *Bacillus stearothermophilus* [54], SoxB from *S. acidocaldarius* [55]

Table 3
Classification of haem–copper oxidases on the basis of the proton channels on subunit I and subunit II features

Oxidase type		Subunit I						Subunit II/FixP[c]					
		Channels						Haem I	Haem II	Cupredoxin domain	Helices	Loop I	Loop II
		'D'[a]			'K'[b]								
		E/Y	Others	K/T	T/S	S/Y	Y						
A1	(e.g., cyt oxidase caa_3)	E	+	K	T	S	Y	–	+	+	2	–	–
	(e.g., cyt oxidase aa_3)							–	–	+	2	–	+
	(e.g., quinol oxidase bo_3)							–	–	+	2	–	–
A2	(e.g., cyt oxidase caa_3)	Y	+	K	T	S	Y	–	+	+	2	–	–
	(e.g., cyt oxidase aa_3)							–	–	+	2	+	–
B	(cyt/quinol oxidases)	–	–	T	S	Y	Y	–	–	+	1	+	–
C	(cyt oxidase)	Y	–	–	S	Y	–	+	+	–	1	–	–

[a]GluI-278, TyrI-256 (*R. marinus*); Others: AspI-124, AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134 and SerI-193.
[b]LysI-354, ThrI-351, SerI-291 and TyrI-280. This tyrosine is covalently bound to one of the histidine ligands of Cu_B (HisI-276).
[c]Dihaemic subunit of type C oxidases.

and aa_3 from *Acidianus ambivalens* [56]. The residues forming the D- and K-channels in type A oxidases are not conserved in these enzymes (Table 2). However, a K-channel homologue is most probably present, with a threonine, a serine and a tyrosine replacing LysI-354, ThrI-351 and SerI-291, respectively (Tables 2 and 3). These enzymes also contain the tyrosine covalently bound to the histidine coordinating Cu_B (TyrI-280). An equivalent for the D-channel has yet to be fully established. The crystallographic structure of the ba_3 oxidase from *T. thermophilus* [23] suggests two other possible proton channels: (i) one, named the Q-pathway, composed of residues GlnI-254, ThrI-396, LeuI-392, SerI-391, ThrI-394, ThrI-81, GlnI-388 and LeuI-387 (*T. thermophilus* numbering), which appears 'behind' the low-spin haem; (ii) another, involving the residues GluI-17, TyrI-91, ThrI-21, SerI-109, GlnI-86, SerI-155, ThrI-156 and GlnI-82 (*T. thermophilus* numbering), that is in a spatial position equivalent to that of the D-channel in the type A oxidases. The functionality of these channels remains to be confirmed by mutagenesis studies. Inspection of the sequence alignment (total of seven sequences) of the type B oxidases shows that none of those residues (or equivalent ones) is common to all of them. However, a double mutant of *P. denitrificans* aa_3 oxidase (PheI-274Thr, GluI-278Ala) shows that ThrI-156 of *T. thermophilus* ba_3 oxidase may indeed be part of a proton channel [57].

A third family of haem–copper oxidases is composed of the cbb_3 oxidases, which for consistency we propose to call type C oxidases. These oxidases seem to have only part of the alternative K-channel conserved, with a serine and a tyrosine in the place of the *P. denitrificans* ThrI-351 and SerI-291 (Fig. 2, Tables 2 and 3). It is remarkable that an equivalent to TyrI-280, the tyrosine covalently bound to a copper histidine ligand, is not present in these enzymes (Fig. 2, Tables 2 and 3). None of the canonical residues of the D-channel is present; there is a tyrosine residue in all cbb_3 oxidases (in a total of 17 in the data bases), with the exception of the one from *Helicobacter pylori*, in the same sequence position as the tyrosine of the YS motif of type A2 oxidases that, as for these oxidases, may be part of the proton pathway.

In summary, the analysis of the proton channels together with the amino acid sequence comparisons clearly allows the recognition of three oxidase families: the mitochondrial-like enzymes (type A), including the two subfamilies A1 (E residue) and A2 (YS motif), the type B enzymes and the type C enzymes (the cbb_3 oxidases) (Table 3). The existence of these three families is fully corroborated by the respective sequence identities and similarities (Table 4), which is reflected in the phylogenetic tree presented in Fig. 3[2].

It is worth stressing that the K-channel is much more conserved than the D-channel, especially the residues equivalent to ThrI-351 and SerI-291. This conservation is mechanistically relevant, as this channel leads directly to the binuclear centre and the reduction of the high-spin haem is coupled to the protonation of a group, most probably a hydroxyl bond to Cu_B. This proton is essential for the scission of the O–O bond [26,39].

2.2. Proton translocation

All terminal oxidases seem to translocate protons. In fact, and contrary to early conservative proposals, members of all three types of terminal oxidases have already been demonstrated to pump protons. Over the last years, evidence has been accumulated that the protons to be translocated as well as those used for the chemical reaction are transported through common channels [20,26,38]. Hence, if the same basic catalytic mechanism is involved in all oxidases, it is to be expected that all oxidases will be proton pumps.

It has been shown that several type A1 oxidases, including the quinol bo_3 oxidase from *E. coli*, are proton pumps with a stoichiometry approaching $1H^+/e$ [58–60]. Type A2 enzymes also pump protons, with the same stoichiometry [48,61], emphasising the

[2] It should be mentioned that the oxidase 1 from *Ae. pernix* [51], which in sequence terms and considering the D-channel is a type A1 oxidase, does not have any of the residues (with the exception of the equivalent to TyrI-280) forming the K-channel, being until now the only enzyme where the residues of this channel or equivalents are not identifiable (Fig. 2). This shows that as more oxidase sequences and structural data will become available, a further subdivision in families of oxidases may become necessary.

Table 4
Values of percentages of identity (top) and similarity (bottom) from the sequence alignment of Fig. 2 (regions with gaps have been removed)

	Type A1								Type A2					Type B								Type C				
	P. den aa_3 1	*E. coli* bo_3 2	*Bo. tau* 3	*B. sub* aa_3 4	*M. tuber* 5	*Ae. pernix* 1 6	*S. acid* SoxM 7	*H. halo* 8	*R. mar* caa_3 9	*A. sp* 10	*Sy. sp* 11	*T. ther* caa_3 12	*D. rad* 13	*Aq. aeol* 1 14	*S. acid* SoxB 15	*Aq. aeol* 2 16	*N. phar* ba_3 17	*T. ther* ba_3 18	*Ae. pernix* 2 19	*B. stear* $ba(o)_3$ 20	*A. amb* aa_3 21	*Br. jap* 22	*H. pylori* 23	*Rhd. caps* 24	*Rh. mel* 25	*Ag. cauld* 26
1		41	57	43	45	39	35	39	44	48	50	45	43	40	18	21	18	21	20	19	19	14	14	13	14	13
		61	77	65	64	60	56	65	66	68	69	67	63	62	38	39	41	40	38	40	38	30	36	33	33	31
2			41	47	43	37	35	34	37	41	40	36	37	37	16	19	18	19	24	18	16	11	14	12	12	11
			61	70	63	61	57	57	57	62	61	61	60	58	38	39	40	38	42	39	34	30	34	32	32	32
3				41	40	36	35	39	41	49	46	38	37	38	19	19	20	20	22	21	17	11	13	12	12	10
				65	61	60	58	66	64	70	69	62	62	61	38	39	43	42	39	42	37	29	34	31	31	30
4					48	40	37	38	38	42	44	40	39	39	19	20	20	22	21	20	21	15	15	16	15	15
					70	63	59	62	64	65	64	65	62	62	35	41	41	42	43	41	38	32	36	34	33	33
5						41	35	42	40	45	46	41	41	41	18	21	19	22	21	22	19	14	15	15	14	12
						60	56	62	63	64	64	63	61	62	35	40	39	41	43	40	36	33	37	35	34	33
6							34	38	36	36	37	36	37	33	18	20	19	25	23	21	18	14	14	13	14	12
							59	59	60	60	58	61	60	58	38	38	40	46	41	39	38	30	35	31	31	30
7								37	36	35	37	35	35	33	21	22	20	24	22	21	18	13	15	14	16	15
								60	59	60	60	58	57	57	39	42	42	44	43	41	37	31	34	31	31	31
8									37	44	45	39	36	34	20	23	23	22	22	22	20	15	17	16	16	15
									63	68	68	61	57	56	36	41	44	43	41	44	39	32	35	33	33	33
9										45	45	40	39	40	17	21	20	22	21	18	18	13	14	15	14	12
										68	68	66	62	61	36	41	42	43	41	41	37	32	36	36	34	33
10											76	41	41	42	17	19	19	18	19	19	19	14	16	16	16	15
											89	65	64	59	39	39	41	40	39	40	38	32	36	34	34	33
11												43	42	40	19	20	19	22	20	21	20	16	17	16	16	16
												67	65	59	38	39	42	42	40	42	40	34	37	35	35	35
12													64	38	18	20	19	20	21	19	19	16	14	16	16	15
													79	59	34	38	40	41	39	38	39	33	36	34	34	33
13														37	17	20	21	22	20	17	22	15	15	14	15	14
														58	32	38	43	40	41	37	41	34	36	33	34	34
14															20	20	18	21	23	17	18	14	16	14	13	12
															39	37	38	38	42	38	36	32	36	31	31	32
15																20	18	19	19	18	22	13	15	16	14	14
																36	35	38	35	37	38	31	35	32	31	31
16																	39	41	45	42	20	14	15	12	13	13
																	58	60	64	62	40	31	34	30	29	29
17																		43	38	44	20	14	16	15	15	15
																		60	63	67	42	33	37	33	32	33
18																			38	45	24	15	18	16	16	16
																			61	67	40	32	35	33	32	32
19																				41	21	15	18	14	15	16
																				65	43	35	37	32	34	33

Table 4 (*continued*).

	Type A1							Type A2					Type B						Type C						
	P. den	*E. coli*	*Ba.*	*B.*	*M.*	*Ae.*	*S. acid*	*H.*	*R. mar*	*A.*	*Sy.*	*T. ther*	*D.*	*Aq.*	*S. acid*	*Aq.*	*N. phar*	*T. ther*	*Ae.*	*B. stear*	*A. amb*	*Br.*	*H.*	*Rhd.*	*Rh.*	*Ag.*
	aa$_3$	*bo*$_3$	*tau*	*sub aa*$_3$	*tuber*	*pernix* 1	SoxM	*halo*	*caa*$_3$	*sp*	*sp*	*caa*$_3$	*rad*	*aeol* 1	SoxB	*aeol* 2	*ba*$_3$	*ba*$_3$	*pernix* 2	*ba*(*o*)$_3$	*aa*$_3$	*jap*	*pylori*	*caps*	*mel*	*cauld*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
20																					21	14	18	14	14	14
																					40	32	36	31	31	30
21																						16	14	16	15	16
																						34	34	31	31	33
22																							38	60	63	68
																							61	72	74	80
23																								41	38	38
																								60	60	59
24																									77	70
																									90	84
25																										76
																										87
26																										

functional substitution of the glutamate (GluI-278) residue by the YS motif.

For the type B oxidases, proton pumping has been demonstrated for the *T. thermophilus ba*$_3$ [62] and *B. stearothermophilus b*(*o*)*a*$_3$ cytochrome oxidases, [63], and for the *A. ambivalens aa*$_3$ quinol oxidase [56]. While for the first two enzymes a stoichiometry of ca. 0.5H$^+$/e was measured, for the *A. ambivalens* enzyme a ratio close to 1 was obtained (as most probably also occurs for the *S. acidocaldarius* SoxB oxidase, see below). The important issue, in the present context, is that all these enzymes are proton pumps. As already noted, none of these enzyme shares common residues in the putative proton channels, besides the equivalent 'K'-channel. For the *A. ambivalens* enzyme, a putative pseudo-D-channel was suggested on the basis of a structural model [56,64]. Interestingly, the hydrophilic part of this channel ends at a glutamate (GluI-80, *A. ambivalens* numbering), located at helix II and not in helix VI as in the type A1 enzymes; this proposal was corroborated by a double mutant in *Rhodobacter sphaeroides aa*$_3$ oxidase mimicking the *A. ambivalens* enzyme, which is fully competent in proton pumping (P. Brzezinski et al., personal communication). For the *S. acidocaldarius* SoxABCD oxidase, a stoichiometry of 1.2H$^+$/e was obtained [65]. Since this enzyme does not contain the 'key' glutamate of the D-channel, it was proposed that a proton pump mechanism was not operative, and instead a Q-cycle should be responsible for proton translocation [65]. However, it is now evident that the glutamate is not essential for proton translocation. Moreover, the above stoichiometry is closer to that of a proton pump (1H$^+$/e) than to that of a Q-cycle mechanism (2H$^+$/e). Hence, most probably this enzyme is also a proton pump. The subunit I of this enzyme, SoxB, has a tyrosine residue equivalent to that of type A2 oxidases, which may be part of the proton channel (Fig. 2).

Proton pumping by the type C (*cbb*$_3$) oxidases has also been demonstrated, first using cells where the genes for other oxidases had been disrupted [66,67], and later with *Bradyrhizobium japonicum cbb*$_3$ oxidase reconstituted in artificial liposomes [68].

In summary, representative members of all families and subfamilies of the haem–copper oxidase superfamily, irrespective of their electron donor, have been shown, unambiguously, to be proton pumps. Con-

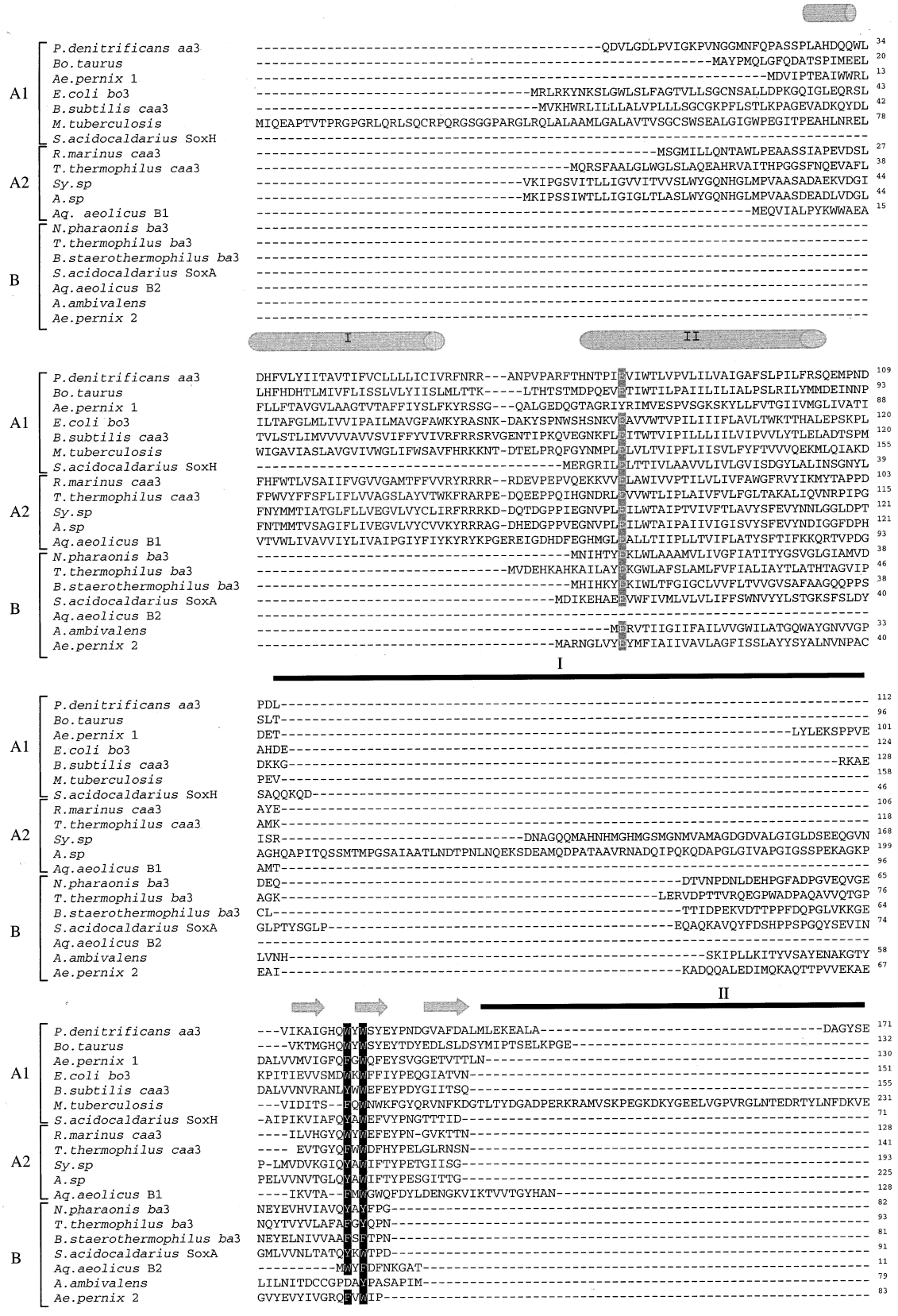
A1
A2
B
P.denitrificans aa3
Bo.taurus
Ae.pernix 1
E.coli bo3
B.subtilis caa3
M.tuberculosis
S.acidocaldarius SoxH
R.marinus caa3
T.thermophilus caa3
Sy.sp
A.sp
Aq. aeolicus B1
N.pharaonis ba3
T.thermophilus ba3
B.staerothermophilus ba3
S.acidocaldarius SoxA
Aq.aeolicus B2
A.ambivalens
Ae.pernix 2
I
II

Fig. 4.

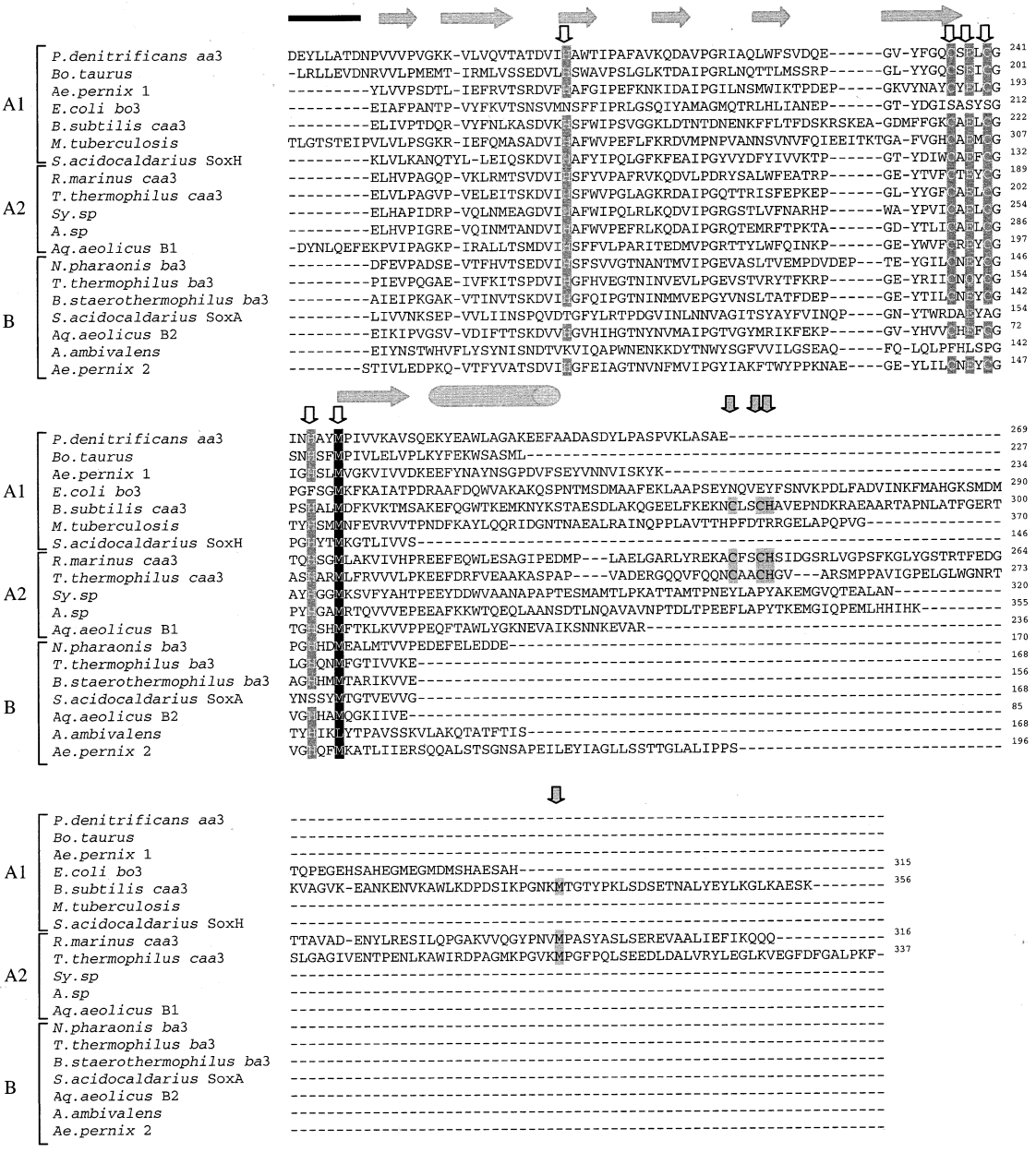

Fig. 4. (*continued*). Amino acid sequence alignment of terminal oxidase subunits II (accession numbers in parentheses). A and B refer to the oxidase families as described in the text. *P., Paracoccus* (1311262A), *E., Escherichia* (A42226), *Bo., Bos* (OBBO2), *Ae., Aeropyrum* (1, Q9YDX7, 2, BAA80721), *B., Bacillus* (*subtilis* caa_3, CAB13363, *stearothermophilus* ba_3, BAA31140), *R., Rhodothermus* (CACO8531), *A., Anabaena* (*Anabaena* sp. strain PCC 7937, CAB10934), *Sy., Synechocystis* (*Synechocystis* sp. strain PCC 6803, S75558), *T., Thermus* (caa_3, AAA27484, ba_3, AAB00369), *Aq., Aquifex* (B1, B70488, B2, D70488), *M., Mycobacterium* (Q10375), *S., Sulfolobus* (SoxH, S56158, SoxA, S21041), *N., Natronobacterium* (CAA71530), *A., Acidianus* (CAA70828). Strictly conserved amino acid residues are shaded in black. Cu_A and haem binding sites are shaded in grey and indicated by the open and the shaded vertical arrows, respectively. Also shaded in grey is GluII-78 (*P. denitrificans* numbering). Transmembrane helices and β-strands are indicated by cylinders and arrows, respectively, on top of the alignment as deduced from the crystal structure of *P. denitrificans* aa_3 oxidase. Alignments were performed as in Fig. 2.

trary to the assumptions of Musser and Chan [16], all terminal oxidases are functionally homologous, thus allowing the establishment of evolutionary links, based on amino acid sequence analysis emphasising the functionally relevant residues. The variability of the proton channels shows that, throughout evolution, multiple structural solutions have been developed to achieve proton conduction and translocation. Even a channel as diverse as that of the *A. ambivalens* oxidase appears to be as competent as those of type A enzymes, since proton uptake occurs with a rate constant comparable to that of type A enzymes [69]. But even if the channels evolved were less efficient, as far as they are kinetically competent they would be operational as the haem–copper oxidases have a proton transfer-controlled reaction cycle, which allows a tight coupling between the redox chemistry and proton pumping (e.g., [4,70]).

3. Subunit II

3.1. Types A and B oxidases

The haem–copper oxidases always contain at least one other subunit, besides subunit I. In types A and B oxidases, this second subunit, named subunit II, is composed of a transmembrane helical domain and a peripheral domain, in the positive (outer or periplasmic) side of the membrane (Fig. 1). The only known exception is the putative subunit II (named B2) from *Aq. aeolicus* [45], which is composed of just the peripheral domain. Fig. 4 shows the alignment of subunit II amino acid sequences from representative examples of types A and B oxidases. Both oxidase families contain members having quinols, cytochromes or HiPIPs as electron donors. It should be noted that a classification based on the type of electron donor does not reflect the sequence and functional features of similar oxidases and thus should not be used.

The transmembrane part of subunits II is composed of one (type B oxidases) or two (type A oxidases) helices (Fig. 1D). Interestingly, the crystal structure of *T. thermophilus* ba_3 oxidase revealed the presence of a third small subunit, having a single transmembrane helix, structurally equivalent to the missing helix of subunit II [23]. It is not yet known if this arrangement is general for the type B enzymes, but at least *N. pharaonis* ba_3 [53], *A. ambivalens* aa_3 [56] and *S. acidocaldarius* SoxABCD [55] oxidases also have small subunits of ca. 50 amino acid residues, which are predicted to form a single transmembrane helix.

With the exception of the oxidases 1 from *Ae. pernix* (which does not have an identifiable K-channel) [51] and 2 from *Aq. aeolicus* (which does not have the transmembrane domain) [45], all other subunits II have in common a glutamate residue (EII-78 in *P. denitrificans*), proposed to be the entry of the K-channel [71], located at the beginning of the single (in type B oxidases) or of the second (in type A oxidases) helix (Fig. 1D).

The peripheral domain has a central cupredoxin-like fold, built by a 10-stranded β-barrel [8–11,23,24] (Fig. 1D). The presence of a metal centre in this domain is related to the type of electron donor: (i) cytochrome *c* and HiPIP oxidases, i.e., oxidases having as electron donor a metalloprotein in the positive side of the membrane (bacterial periplasm or mitochondrial intermembrane space), contain a binuclear copper centre, Cu_A, which receives electrons from cytochrome *c*; (ii) quinol oxidases lack this centre, but the fold is conserved, as deduced from the crystal structures of the *E. coli* bo_3 oxidase [24,72], amino acid sequence comparisons and structure predictions of the other enzymes (our unpublished data). Several type A oxidases, besides the Cu_A centre, also contain a haem centre in their C-termini (Table 3), which forms a cytochrome domain, including the haem attachment signature –CxxCH–; these enzymes are generally named caa_3 oxidases (Fig. 5). The ligands to the Cu_A site (HisII-181, CysII-216, GluII-218, CysII-220, HisII-224 and MetII-227) are conserved among type A and B cytochrome oxidases, with the exception of the ba_3 oxidase from *T. thermophilus*, in which the residue equivalent to GluII-218 is substituted by a glutamine. In all known cytochrome oxidases, this centre is binuclear, which has been explained by the unusually low electron transfer reorganisation energy at this site, such that electron transfer is not the rate-limiting step [73].

A close examination of the three-dimensional structures and amino acid sequences of subunits II reveals the presence of loops of different sizes between key domains, that are relevant in both func-

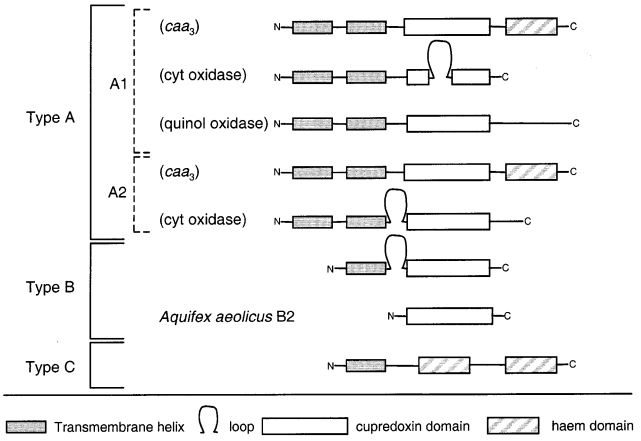

Fig. 5. Schematic representation of the different domains of representative subunits II from terminal oxidases of the three families.

tional and evolutionary terms (Figs. 1D and 5). In type A1 cytochrome oxidases, excluding the caa_3 oxidases, a loop (loop II, Figs. 1D and 5) is inserted between strictly conserved aromatic amino acids (TrpII-121, TrpII-123, *P. denitrificans*), and some residues before the first ligand of Cu_A (Figs. 4 and 5, Table 3). This loop contains several amino acids involved in the interaction with cytochrome *c* [74,75], thus explaining why it is not present in quinol and caa_3 oxidases (Figs. 4 and 5). In this last case, this absence is probably related to the presence of the haem domain. Homology modelling studies performed with the subunit II of *R. marinus* oxidase confirm the absence of this loop, as in the structures of the Cu_A-engineered *E. coli* bo_3 quinol oxidase [72] and *T. thermophilus* ba_3 oxidase [23], suggesting that the haem domain lays just above the copper centre in the spatial place of the loop [49]. This loop is also absent in all type B oxidases. Again, this may be functionally related to the interaction with its electron donor: for the *T. thermophilus* ba_3 cytochrome oxidase its interaction with *T. thermophilus* cytochrome c_{552} was shown to have an electrostatic behaviour quite distinct from that of the *P. denitrificans* aa_3 and mitochondrial oxidases with the respective cytochromes *c* [76].

For the three known cyanobacterial oxidases, instead of loop II another loop is present (loop I, Figs. 1D and 5) inserted in a different position, immediately after the transmembrane helix and before the cupredoxin-like domain. The electrostatic interaction between the cyanobacterial oxidases and cytochrome c_6 is similar to that of the *T. thermophilus* ba_3 enzyme with cytochrome c_{552} [77], suggesting the functional relevance of this loop. A loop in the same position, but shorter, is present in type B cytochrome or quinol oxidases (Figs. 1D and 5, Table 3).

SoxH (subunit II of *S. acidocaldarius* SoxM, a member of the type A1 oxidases) does not contain any loop, which can be explained by the fact that it receives electrons from another subunit of the oxidase complex [78].

In conclusion, the three family types, defined on the basis of subunit I characteristics, also present distinctive features in subunit II, typical of each family (Table 3, Fig. 5).

3.2. Type C oxidases

The members of the cbb_3 family, apart from subunit I, have two other subunits containing C-type haems: a monohaemic and a dihaemic cytochrome. They do not show significant amino acid similarities among each other, or with other known cytochromes (as also observed for the haem C domain in the caa_3 oxidases [49]). However, a possible relationship between the dihaemic subunit (FixP) and the caa_3 oxidases subunit II can be observed (Fig. 6). The sequences are all approximately the same size, especially in the predicted peripheral domains. Surprisingly, the C-termini, including the haem binding site, are reasonably similar, especially in the region close to the sixth ligand methionine, suggesting a possible evolutionary link between these cytochrome domains. Also interestingly, the binding site of the first haem of FixP seems to be part of the binding site of the Cu_A centre of types A and B oxidases: the axial ligands of the haem, the histidine and the methionine, are ligands of the copper centre (Figs. 5 and 6). However, the predicted secondary structure is different in these regions: the first haem domain is predicted to be composed of small helices connected by different loops, while the Cu_A domain has a cupredoxin-like fold.

4. Evolution of terminal oxidases

In the previous sections it was shown that terminal

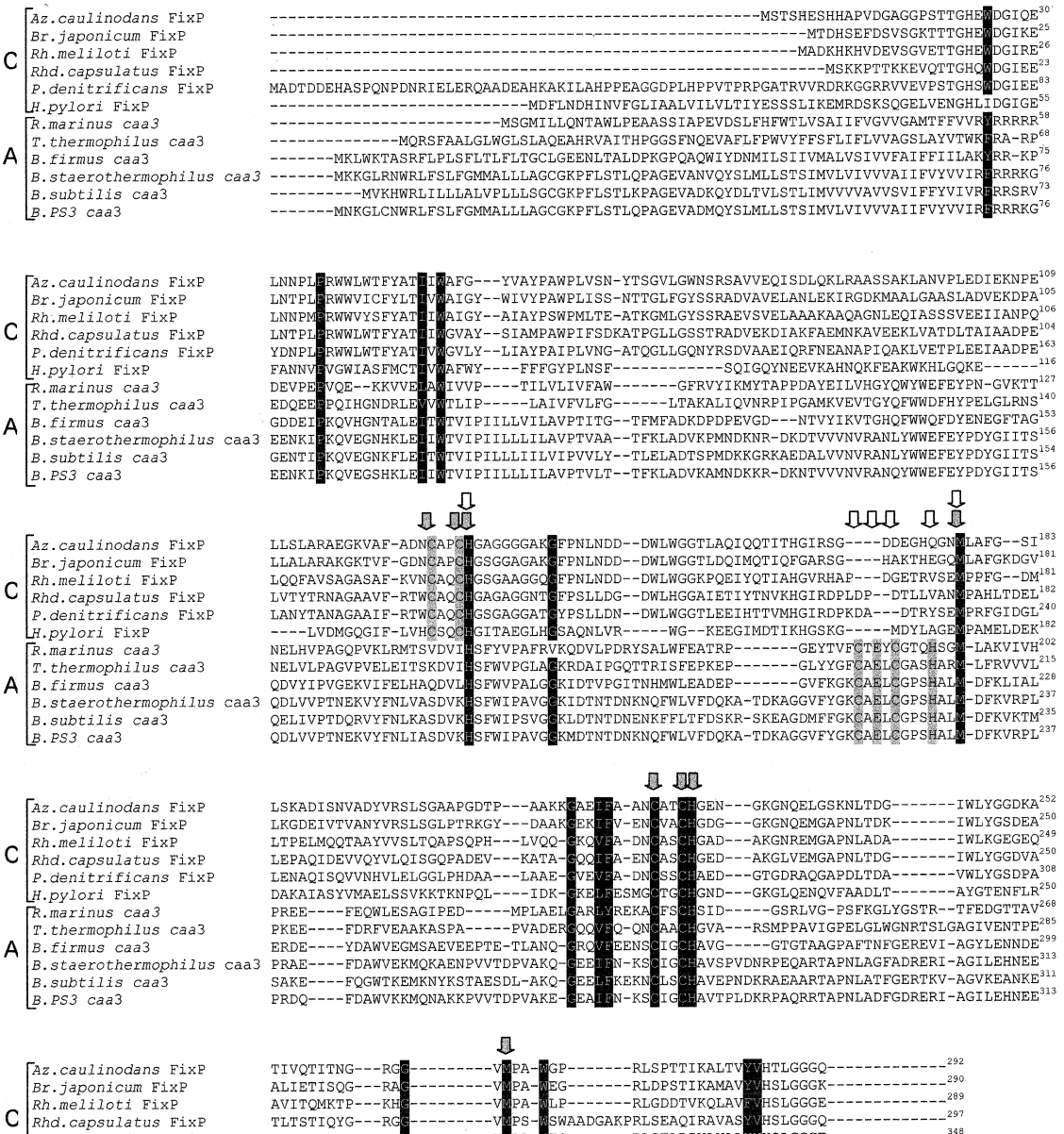

Fig. 6. Amino acid sequence alignment of caa_3 oxidase subunit II and dihaemic subunit (FixP) of cbb_3 oxidases (accession numbers in parentheses). A and C refer to the oxidase families as described in the text. *Az., Azorhizobium* (D55582), *Br., Bradyrhizobium* (AAC15891), *Rh., Rhizobium* (S39991), *Rhd., Rhodobacter* (AAC46111), *P., Paracoccus* (S77598), *H., Helicobacter* (AADO77216), *R., Rhodothermus* (CACO8531), *T., Thermus* (caa_3, AAA27484), *B., Bacillus* (*firmus* caa_3, Q04441, *stearothermophilus* caa_3, BAA11111, *subtilis* caa_3, CAB13363, PS3, Q03438). Strictly conserved amino acid residues are shaded in black. Cu_A and haem binding sites are shaded in grey and indicated by the open and the shaded vertical arrows, respectively. Alignments were performed as in Fig. 2.

oxidases from all three families, apart from reducing dioxygen to water, are proton pumps. Thus, besides the conservation of the metal centres and the overall structure, the oxidases from the haem–copper superfamily are functionally homologous. Hence, all the evolutionary considerations based on amino acid sequence alignments, always taking into account the relevant residues, are valid.

4.1. Oxidase distribution among the three domains of life

Mitochondria contain only type A1 oxidases, while prokaryotes contain all oxidase types, but not uniformly distributed (Table 5 Fig. 7). The first observation is that all families occur in Bacteria, while in Archaea (Crenarchaeota and Euryarchaeota) only types A1 and B have been detected until now. Type C oxidases (cbb_3 enzymes) have been found in only two bacterial groups – purple bacteria and *Flexibacter*, *Bacteroides* and *Cytophaga* (*R. marinus* [79]). Interestingly, these last enzymes, reported to have a higher oxygen affinity [80–82], are present in many human pathogens which thrive under microaerophilic conditions.

Within the Bacteria domain, a clear division appears between the two type A subfamilies: the type A1 enzymes, containing the helix VI glutamate residue of the D-channel (GluI-278, *P. denitrificans*), occur only in the upper bacterial branches (Gram-positive and purple bacteria); in contrast, type A2 enzymes, lacking that glutamate and having instead

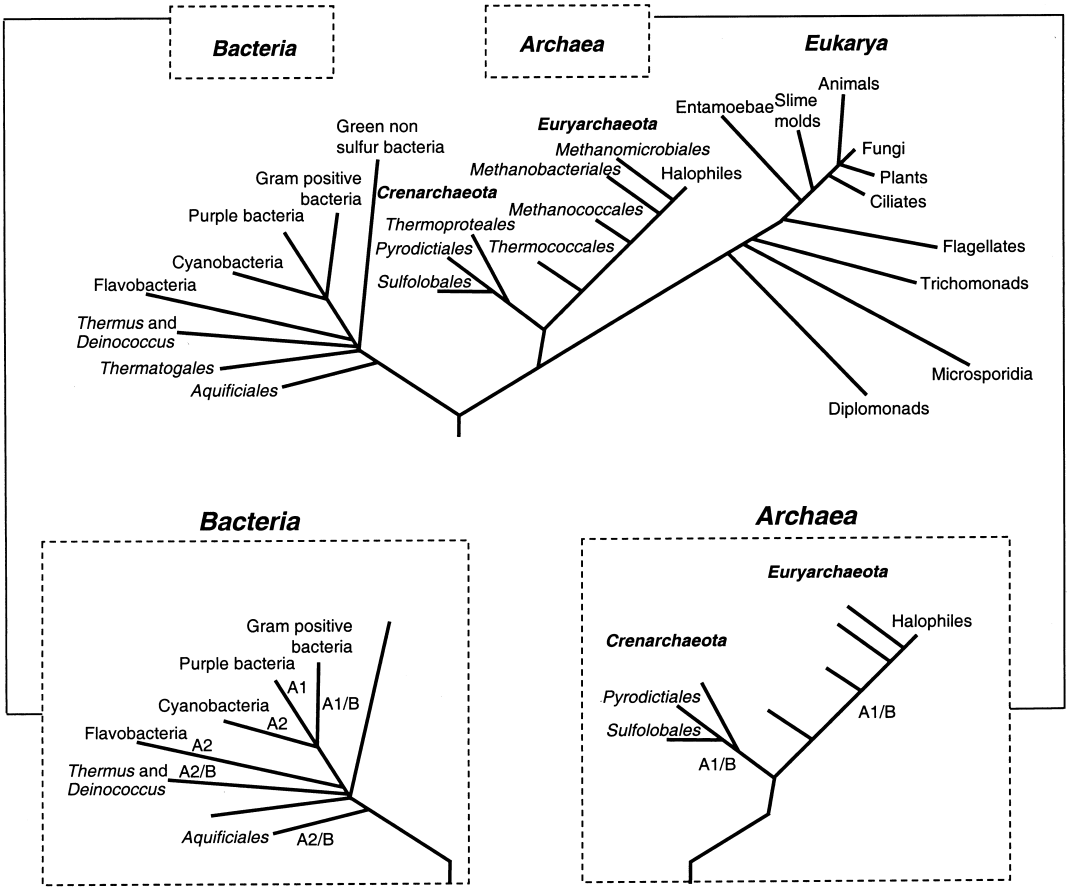

Fig. 7. 16S rRNA phylogenetic tree (adapted from [91]), showing the distribution of A1-, A2- and B-type oxidases, among the different branches of the Bacteria and Archaea domains. In the Bacteria domain (bottom left) the YS motif (type A2) is present in oxidases from bacteria belonging to the deepest branches of this domain, while the glutamate-containing oxidases (type A1) are exclusive of the purple and Gram-positive bacteria. The type B oxidases are present in all bacterial branches with the exception of the purple bacteria group. In the Archaea domain (bottom right) type A1 and B oxidases are present in organisms from both Crenarchaeota and Euryarchaeota subdomains. The strictly anaerobic branches have been removed.

Table 5
Distribution of the three types of haem–copper oxidases among the different domains of life

Domain	Subdomain	Group/order/genus	Oxidase type
Bacteria		Aquificiales	A2 and B
		Thermus and *Deinococcus*	A2 and B
		Cyanobacteria	A2
		Flexibacter, *Bacteroides* and *Cytophaga*	A2 and C
		Gram-positive	A1 and B
		Purple bacteria	A1 and C
Eukarya			A1
Archaea	Crenarchaeota	*Sulfolobales, Pyrodictiales*	A1 and B
	Euryarchaeota	Halophiles	A1 and B

the YS motif in the same helix, are present in organisms that belong to the deepest branches of the 16S rRNA phylogenetic tree (Fig. 7). Therefore, we propose that the type A1 oxidases evolved from the type A2 ones.

The endosymbiotic theory explains the presence of mitochondria and thus the ability of respiration using molecular oxygen as the last electron acceptor in the Eukarya domain. As mitochondria are placed in the phylogenetic tree as a branch diverging just before the purple bacteria, it is not surprising that mitochondrial oxidases are very similar to type A1 cytochrome oxidases from purple bacteria.

In the Archaea domain, only types A1 and B oxidases are present, in both Crenarchaeota and Euryarchaeota subdomains. Most strikingly, all archaeal oxidases have in general a higher amino acid identity (and similarity) with the bacterial enzymes, especially from Gram-positive bacteria, than with other archaeal oxidases (Table 4), in contrast to what is observed within the bacterial enzymes. This observation suggests that the archaeal enzymes have not evolved from a common archaeal ancestor. We propose that the members of the Archaea domain acquired terminal oxidases by gene transfer from the Gram-positive bacteria. This hypothesis implies that the last common ancestor, before the divergence between Archaea and Bacteria, did not have a terminal oxidase of the haem–copper type, which would have evolved first in the bacterial branch. This proposal diverges from the hypothesis of Castresana and Saraste [14] who, considering the fact that bacteria and archaea have terminal oxidases, suggested that these enzymes had a monophyletic origin in a last common ancestor of both prokaryotic domains. The data here presented are also consistent with a monophyletic origin for oxidases, but confine it to a common bacterial ancestor. The same authors also suggested that the cbb_3 oxidases (type C) are closest to the so-called uroxidase [83,84]. As mentioned above, these oxidases are only present in purple bacteria and *Flexibacter*, *Bacteroides* and *Cytophaga* groups and are neither observed in the deepest bacterial branches nor in the Archaea domain. This observation makes it difficult to sustain a close relation between the type C oxidases and the uroxidase. Nevertheless, the proposal that terminal oxidases existed before the development of oxygenic photosynthesis [13] may still hold, since terminal oxidases are present in bacterial branches deeper than Cyanobacteria, such as Aquificiales and *Thermus* and *Deinococcus*.

Castresana and Saraste [14] also proposed that the evolution of haem–copper reductases was linked to enzymes involved in the nitrogen cycle, especially N_2O and NO reductases (NOR). This hypothesis was based on the observations that (i) N_2O reductase contains at the C-terminus a cupredoxin-like domain quite similar to that of subunit II from type A and B haem–copper oxidases [85]; (ii) subunit I from NOR has a general predicted structure reminiscent of subunit I from haem–copper oxidases, including the six histidines binding the metal centres; (iii) the other subunit of NOR resembles the monohaemic subunit of type C oxidases. It was then proposed that haem–copper oxidases evolved from NOR, the cbb_3 enzymes being the first oxidases to appear by incorporation of the Cu_B site. NORs have never been observed in the deepest bacterial branches, and their presence has been reported only in the purple and Gram-positive bacteria groups [86]. However, the gene encoding one of its subunits (cytochrome *b*) is present in the genome of *Synechocystis* sp. strain

PCC6803 [87]; NOR is also predicted to exist in the Archaeon *Pyrobaculum aerophilum* [88] (which may have acquired it by gene transfer from Gram-positive bacteria, in the same way that we suggested for the oxygen reductases). Hence, as above discussed for the cbb_3 oxidases, it becomes difficult to envisage these enzymes at the origin of respiration. Nevertheless, there is certainly an evolutionary link between the enzymes of these two metabolic processes (oxygen or nitrogen respiration), but it is not possible with the present knowledge to decide which enzymes evolved first.

4.2. A parallel evolution of subunits I and II

All haem–copper oxidases, besides the core subunit I, have at least another subunit which appears to be quite conserved throughout evolution, either in type A and B oxidases, or in type C ones. Furthermore, we showed in Sections 2 and 3 that each oxidase family can be identified by features from either subunit (Table 3). Therefore, we proceed to analyse a possible co-evolution of both subunits. Putting together all the discussed common features of terminal oxidases and their presence in organisms distributed among the 16S rRNA phylogenetic tree, evolutionary

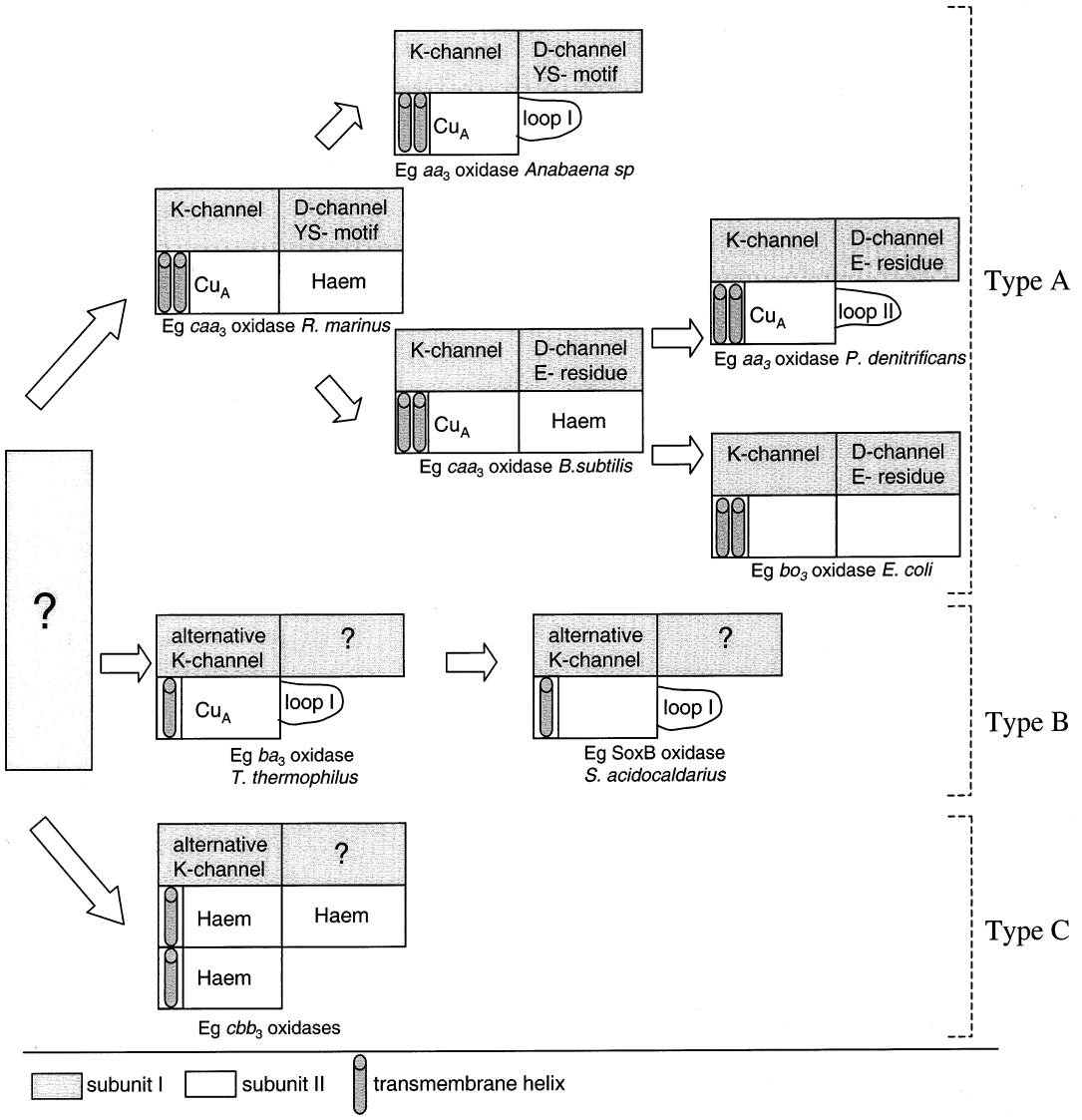

Fig. 8. Parallel evolution of subunits I and II (the minimal functional unit) of terminal oxidases from the three families: types A, B and C oxidases.

relationships among the oxidases were tentatively established as described in Fig. 8. Three parallel evolutionary lines are identified coincident with the three families proposed. With the available data, an evolutionary relation between the first members of each family is not yet possible to define.

Regarding type A oxidases and considering the fact that the YS motif-containing ones (type A2) are ancestral to the E residue-containing oxidases (type A1), and that the genera *Thermus* and *Deinococcus* (containing type A2 caa_3 oxidases) are in a deeper branch than cyanobacteria (containing type A2 aa_3 oxidases), it is suggested that in this evolutionary line the first known oxidase was a type A2 caa_3 oxidase. Two independent occurrences gave rise to the terminal oxidases of cyanobacteria and of the caa_3 oxidases from *Bacilli*. In the case of the cyanobacterial enzymes the event was the loss of the C-terminal domain of the subunit II from the first oxidase and the insertion of a loop (loop I, Figs. 5 and 8) between the transmembrane domain and the conserved aromatic residues (equivalent to TrpII-121 and TrpII-123, see above, Figs. 4 and 5). In the case of *Bacilli* the change observed is the functional substitution of the YS motif of the D-channel for the E residue in subunit I. From this last oxidase an enzyme like the SoxM from *S. acidocaldarius* and like the bo_3 oxidase from *E. coli* could have evolved, by the loss of the C-terminal domain of subunit II or by the loss of the prosthetic groups of the same subunit, respectively. The most known oxidases, such as those from the purple bacteria and mitochondria, could have evolved from a SoxM-like oxidase in which a loop was inserted after the aromatic pattern and before the Cu_A binding site (loop II, Figs. 1D, 4 and 5).

The occurrence of quinol oxidases in the family of the type B oxidases could have happened in the same way as in type A oxidases, i.e., by the loss of the prosthetic groups of subunit II. This implies that quinol oxidases did not have a monophyletic origin, and evolved at least twice, in the type A and type B families. Supporting this hypothesis is the observation that the quinol binding site suggested for the *E. coli* bo_3 oxidase (ArgI-71, AspI-75, HisI-98, GlnI-101, *E. coli* numbering) [24] is conserved in ubiquinol oxidases from type A, whereas in type A menaquinol oxidases the equivalent to Gln-101 is substituted by an aspartate residue (our unpublished results). Nevertheless, the proposed quinol binding site is quite conserved in type A and absent in the known type B quinol oxidases, such as the oxidases aa_3 from *A. ambivalens* and SoxABCD from *S. acidocaldarius*.

5. Conclusion

In summary, on the basis of a comprehensive analysis of all amino acid sequences available for haem–copper oxidases, together with the structural and functional data for these enzymes, it is possible to establish the minimal common features for this enzyme superfamily. This approach allowed us to define three families of oxidases: type A, type B and type C. Type A family can be further divided into two subfamilies: type A1 (E-containing oxidases) and type A2 (YS-containing oxidases). The correlation of this classification with the organismal 16S rRNA phylogeny made possible the establishment of several evolutionary steps. It is observed that type A2 oxidases are ancestor to type A1. Also, the data point to the development of aerobic respiration first in the Bacterial domain, the Archaea having acquired this capability through multiple events of inter-species gene transfer, probably from Gram-positive bacteria (a close relation between Archaea and Gram-positive bacteria has been previously proposed on the basis of analyses of several protein families [89]). The three oxidase families here defined can also be identified by common features of their subunits II, making it possible to establish a parallel evolution of the core subunits (subunits I and II).

The 'minimal' functional features for the haem–copper superfamily have interesting implications for the molecular mechanisms of these enzymes, and for the coupling elements between the chemical/redox steps and the pumping of protons. It is clear that protonatable residues are not essential for efficient proton transfer along the intra-protein pathways. There remain as the only common features the metal centres, the low-spin haem and the haem–copper centre and possibly, in some steps of the catalytic reaction, protein exogenous ligands to the metal centres, together with a few amino acid residues in close interaction with the metal ligands and the haems.

Acknowledgements

We would like to thank Matti Saraste for stimulating discussions and suggestions, Cláudio M. Gomes and Cláudio M. Soares for critical reading of the manuscript and Sorel T. Fitz-Gibbon for providing the *Pyrobaculum aerophilum* oxidase sequence before publication. M.M.P. and M.S. are recipients of grants from the Praxis XXI programme (BPD/22054/99 and BPD/3382/96).

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