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Review

# A novel scenario for the evolution of haem-copper oxygen reductases

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#### 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<sub>3</sub>-like oxidases) and (iii) type C (cbb<sub>3</sub>-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 cbb3 oxidases and subunit II of caa3 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. © 2001 Elsevier Science B.V. All rights reserved.

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 termi-

\* Corresponding author. Fax: +351-21-4428766; E-mail: mpereira@itqb.unl.pt nal 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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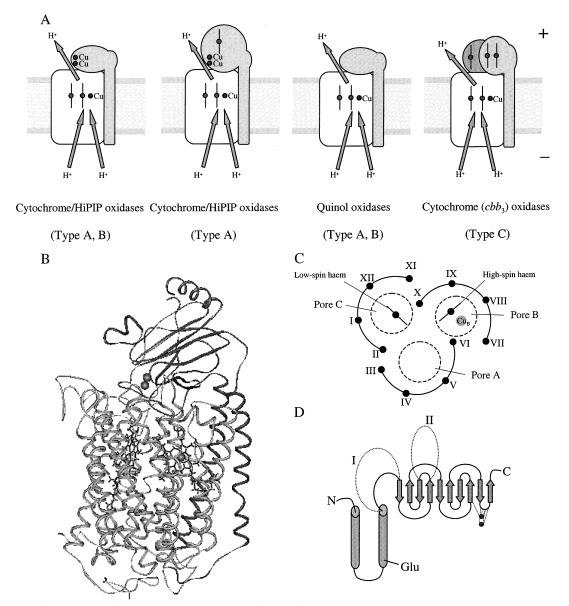


Fig. 1. (A) Schematic representation of the main types of terminal oxidases. Proton pathways are schematically represented by arrows. (B) Crystal structure of subunits I (light grey) and II (dark grey) from *P. denitrificans aa*<sub>3</sub> oxidase (PDB code 1AR1). (C) Schematic view of subunit I of 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 sim-

ilar 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 (<u>high potential iron-sulfur pro-</u> tein) oxidases this domain contains a mixed-valence binuclear copper site (Cu<sub>A</sub>), while in quinol oxidases

|     | P.denitrificans aa3                     |  |                        | MADAAVHGHGT                                       | HHDTRGFFTRWFMSTNHKDI <sup>31</sup>  |
|-----|---|--|------------------------|---|---|
|     | E.coli bo3                              | MFGF   | KI.SLDAVPFHEPTVMVTTAG  | TILGGLALVGLITY                                    | FGKWTYLWKEWLTSVDHKRL <sup>57</sup>  |
|     | Bo.taurus                               |  |                        |   | MFINRWLFSTNHKDI <sup>15</sup>   |
|     | B.subtilis caa3                         |  |                        |   |   |
| A1  | M.tuberculosis                          |  |                        |   |   |
|     | Ae.pernix 1                             |  |                        |   |   |
|     | S.acidocaldarius SoxM                   |  |                        |   |   |
|     | H.halobium                              | MATAASSITLTVLMGVLI                               | LVGVVAVLARLEDWRSYTPL   | SDVGGGLGERTGYI                                    | THEEKPGGIIRWFTTVDHKDI   |
|     | R.marinus caa3                          |  |                        |   |   |
|     | A.sp                                    |  |                        |   |   |
|     | Sy.sp                                   |  |                        |   |   |
| A2  | T.thermophilus caa3<br>D.radiodurans    |  |                        |   |   |
|     | Ag.aeolicus Al                          |  |                        |   |   |
|     | S.acidocaldarius SoxB                   |  |                        |   |   |
|     | Aq.aeolicus A2                          |  |                        |   | MWVMQVSNAIKFIILT <sup>16</sup>  |
|     | N.pharaonis ba3                         |  |                        |   |   |
|     | T.thermophilus ba3                      |  |                        |   |   |
| B   | Ae.pernix 2                             |  |                        |   | MSGGKVSFSAEDRFTKV'  |
|     | B.stearothermophilus ba(0)3             |  |                        |   | MVQPLEKVDRRDAKL <sup>13</sup>   |
|     | A.ambivalens aa3                        |  |                        |   |   |
|     | H.pylori cbb3                           |  |                        |   |   |
| С   | Br.japonicum cbb3                       | -MSQPSISKSMTIGESGLAVVFA<br>MWDYVKLVALGVVAAIAA    | AATAFLOVIAAAKALDAPFA   | Γ ΠΑΑΔΟΑΑΑΟ ΥΑΑΥ                                  | CDDKDSKDEVEDG-VTRAGV <sup>70</sup>  |
|     | Rhd.capsulatus cbb3<br>Rh.meliloti cbb3 |  | SUGAFLALVGAGLAODRLE    | CAHMWULFFALLAG                                    | GDI KI SKEETI DO VINGOV<br>GTLVLMRRVDFRPA-VAGHPG <sup>62</sup>            |
|     | Az caudinolans cbb3                     | MSIVQTPAKRMTGGELGLILVFA                          | AALGEESTVVAAKAYTPEYA   | FHAYLFAAASIAT                                     | /FVIGNRYMDRPAELPPOTID <sup>76</sup>                                       |
|     | - Az. Caldinolans CDDS                  | MSIVQIIARRAIGOBBOBIEVII                          |                        |   |   |
|     |   | I  |                        |   | II  |
|     | P.denitrificans aa3                     | GILYLFTAGIVGLISVCFTVYM                           | MELQHPGVQYMCLEGARLI    | ADASAECTPNGHL                                     | NVMITYHGVLMMFFVVIPAL <sup>107</sup>                                       |
|     | E.coli bo3                              | GTMVTTVATVMLLRGFADATMM                           | SOOALASAGEAGEL         | PPHH  | DOIFTABGVIMIFFVAMPFV  |
|     | Bo.taurus                               | OTTALECAWACMUCTALSLLT                            | AFLCOPCTLLC            | DDOT  | NVVVTABAFVMIFFMVMPIM'"  |
|     | B.subtilis caa3                         | AILYLVAGGFFFLVGGIEAMFI                           | IQLAKPENAFL            | SAQA  | NEVMTMHGTTMIFLAAMPLL <sup>86</sup>  |
| A1  | M.tuberculosis                          | GIMYCVACISFFFIGGLLALLM                           | TELAAPGLQFL            | SNEQ  | NQLFTMHGTIMLLFYATPIV <sup>33</sup>  |
|     | Ae.pernix 1                             | GLLYLVTSIAFLLIAGSLALLF                           | VQLAIPKSNFL            | TGDA  | YEAVTVIGLIMLLWFASPFA <sup>**</sup>  |
|     | S.acidocaldarius SoxM                   | GQMYIVLGIVALIIGSVNAALI                           | DQLSFNNLNAV            | D   | YDAVTLHGIFMIFFVVMPLS <sup>12</sup>  |
|     | H.halobium                              | GILYGVYGTIAFAWGGVSVLLM<br>GILYLISVVFFFIVGGILAVLI | TELATSSETLI            | SPSL  | NGLLTSIG-TIMLFLFGTPM  |
|     | R.marinus caa3                          | GLYLISVVFFFIVGGILAVLI<br>GLQYLVTSFIFYCIGGVMADLV  | LELLQPGQTLM            | CDEV  | MCIETINGAIMIFUEINIVEAC <sup>96</sup>                                      |
|     | A.sp                                    | GIQYLVTSFIFYCIGGVMADLV<br>GIQYLVTSFLFFFIGGSFAEAM |                        | SPEV  | NOLMTLHGTIMIFLWIVPAG <sup>89</sup>  |
|     | Sy.sp<br>T.thermophilus caa3            | GLMYTATAFFAFALAGVFSLLI                           |                        | TGEO  | NOTLTLEGATMLFFFIIOAG <sup>86</sup>  |
| A2  | D.radiodurans                           | GLLYIIVSILGFCLGGLLALAI                           | VOLAL PEOTLE           | VGTT  | NOVLTMHAAIMLFFFLIPLG <sup>94</sup>  |
|     | Ag.aeolicus Al                          |  | MPGMOVV                | DFKT  | NYLLTG GVGMLFWWAIAAH <sup>32</sup>  |
|     | S.acidocaldarius SoxB                   | WWI.VTTCSTEWI.CVI.GTAAMNI                        | TFLTYDONSPN            | VGEL  | YSALTINGWAAMIAFVPMAA <sup>78</sup>  |
|     | Aq.aeolicus A2                          | ETTERTITUECTVHCVMOVEV                            | SGITKAESEL             | GID   | YOGLTLEGVINVIVYTTIFI'   |
|     | N.pharaonis ba3                         | AFFTSFAALAVGAALGLTOVLH                           | VDVVRFTDSAK            |   | YDVLTLHGVLLVITFTIFFL <sup>72</sup>  |
|     | T.thermophilus ba3                      | TUVELVICELALIVCELEGPEO                           | ALNYGNUDAYPLLKRLLP     | FVOS  | YOGLTLEGVLNAIVFTOLFA°   |
| в   | Ae.pernix 2                             | TMLVAFLMLPLGGLEGTLOLEK                           | TPNFPDPVSDS            | T   | YTALTG GVVLAIVFTTFFI''  |
|     | B.stearothermophilus ba(0)3             | ALAHLFVAFIALGLGGFAGLLQ                           | TLVRSGKFELPGG          | IS  | YTILTTHGVLLGLVLTTFFI'   |
|     | A.ambivalens aa3                        | VMAMLVMGVIWGLLGVIDSLMV                           | RIQESTWGTYGVLAM        | TSQE  | YAGITLHAERDLFGFAEQVE  |
|     | H.pylori cbb3                           | FLYAMVGFGIVGMLIGIVLAFE<br>GRPNYNMGPIKFSSFMAMFWGI | LSFPNLNYIAGEYG         |   |   |
| С   | Br.japonicum cbb3                       | IATTFWGIVGFLVAVVIAFQLA                           | AGFLVGLIIASQLAWPALNF   | DLPWIS  | GRURPLETSAVIFAFGGNAL <sup>127</sup>                                       |
|     | Rhd.capsulatus cbb3<br>Rh.meliloti cbb3 | RRREYFDEVVKYGVVATVFWGV                           | VCFLVCVAVALOLAFPELNV   | EPWFN   | GRVRPL TSAVIFAFGGNAL <sup>130</sup>                                       |
|     | Az.caudinolans cbb3                     | GKPNYNMAPVKVGTLLAVFWGI                           | AGFLIGVITALOMAYPLENE   | DLPWIS  | GRLRPLHTSAVIFAFGGNVL <sup>145</sup>                                       |
|     | Az. Caudinorans Cobs                    |  |                        |   | a   |
|     |   | Constant and the constant ( )                    | ( III                  |   |   |
|     |   | D D  | D                      |   | 170   |
|     | P.denitrificans aa3                     | FGGFGNYFMPLHIGAPD                                | MAFPRLNNLSYWMYVCGV     | ALGVASLLAPGGNI                                    | DQMGSGVGWVLYPPLSTT  |
|     | E.coli bo3                              | -IGLMNLVVPLQIGARD                                | VAFPFLNNLSFWFTVVGV     | ILVNVSLGV   | -GEFAQTGWLAYPPLSGI-EY   |
|     | Bo.taurus                               | IGGFGNWLVPLMIGAPD                                | MAFPRMNNMSFWLLPPSF     | LLLASSMVEAG                                       | AGTGWTVYPPLAG-NLA   |
| Al  | B.subtilis caa3                         | -FALMNAVVPLQIGARD                                | VSFPFLNALGFWLFFFGG     | TLP PUPER AND | -GGAPDAGWTSYASLSLH <sup>146</sup><br>-GGAADFGWTAYTPLTDA-IH <sup>162</sup> |
| AI  | M.tuberculosis                          | -FGFANLVLPLQIGAPD                                | VAFPRLNAFSFWLFVFGA     | TIGAAGFITP  | APNVGWTLYAPLTAR-IY  |
|     | Ae.pernix 1<br>S.acidocaldarius SoxM    | -FGLANYIVPLQIGARD                                | LAF PRENALS I WEI BESG | TLAATSPLL   | GAVDLGWYMYAPLSVETTV <sup>137</sup>  |
|     | H.halobium                              | TAAFGNVETPLLTDADD                                | MAEPRINATAEWLLPPGA     | ILIWSGFLIPG                                       | -IATAOTSWTMYTPLSLQ <sup>192</sup>   |
|     | R.marinus caa3                          | PAVIGNFALPIMIGAKD                                | VAEPRINIASWYIEWIGA     | LTMLVGIVTSG                                       | LDTGWTFYTPYST <sup>155</sup>  |
|     | A.sp                                    | -AGEANVLIPLMIGARD                                | MAEPRINAVAEWMIP-PA     | GLLLIASLVV  | -GDAPDAGWTSYPPLSLV***   |
|     | Sy.sp                                   | -AAFANYLIPLMVGTED                                | MAFPRLNAVAFWLTP-PG     | GILLISSFFV  | GAPQAGWTSYPPLSLL***   |
| 4.2 | T.thermophilus caa3                     | LTGFGNFVVPLMLGARD                                | VALPRVNAFSYWAFLGAI     | VLALMSYFFP  | -GGAPSVGWTFYYPFSAQ***   |
| A2  | D.radiodurans                           | LFGFGNYFLPLQLGVRD                                | VALPRLNTFAVWLFIASL     | ILVVLGLFN   | -GGAPSVGWTFYYPLTMDG <sup>156</sup>  |
|     | Aq.aeolicus Al                          | IGGFGNFLLPLMIGAKD                                | VAFPRLNALSWWAFFAAN     | VLFLMTLIP   | -GNHIMIMWIGY PPESLN <sup>22</sup>   |
|     | S.acidocaldarius SoxB                   | AAVIGFS-LYKSKLSI                                 | IHTKQMAIFFWLSN         | IVLLGIAMA   | GSPDMGWYMYPPLAIES <sup>132</sup>  |
|     | Aq.aeolicus A2                          | VGFSNALVAYSLKKPLR                                | EKVQWIALGMMV1GT        | DMAGGI.TDCTEM                                     | GRATVLYTFYPPLI <sup>128</sup><br>SAAVLFTFYAPMQ <sup>135</sup>             |
|     | N.pharaonis ba3                         | ON TMUVI DAREI MMP                               |                        | WAT PLIAN   | EATVLYTFYPPLK <sup>140</sup>  |
| в   | T.thermophilus ba3<br>Ae.pernix 2       | MGTSA FTVSRELKTN                                 | FSRLALNI.SLGLALLGT     | WLAAVAILS   | GOAKVLYTFYPPLK  |
| 5   | B.stearothermophilus ba(0)3             | TGEOFAAVSRTAGAET-                                | DSTRRVGWIGEWIMTIGT     | AMSAFFTLT   | GOAAVLYTFYAPLO  |
|     | A.ambivalens aa3                        | FALET VETTKLLNLOPRA                              | KWILLNTAFTAINTSMMFMEG  | PTVVFPTFNDN                                       | YFSATDWYYISPMG  |
|     | H.pylori cbb3                           | MASHVVICOBVIKITVH-                               | -OHPFLKTVGLLHFWLWTTL   | LTLGVISLFAG                                       | LTOSKEYAELM   |
| ~   | Br.japoničum cbb3                       | TATCEVINOKSCRUB                                  | LAGDLAPWEV///GYNEE     | TLVAGT  |   |
| С   | Rhd.capsulatus cbb3                     | TACAE VINIOPTCAAP                                | -LECCTALCWEVEWGWOLTT   | WTAAT   |   |
|     | Rh.meliloti cbb3                        | TATCEVINORTCRAR                                  | -LEGG-DLGWEVEWGYOLET   | VLAAS   |   |
|     | └ Az.caudinolans cbb3                   | IATSFYVVQRTSHAR                                  | -LAGY-LAPWFVVLGYNFFI   | V1AGT   | 183   |
|     |   | Fig  | 2                      |   |   |

|    |   | ( IV  |  | V  |  |
|----|---|---|--|--|--|
| Ē  | P.denitrificans aa3   | D<br>EAGYSMDLAIFAVHVSGASSILGAI  | INIITTFLNMRAPGMTLF                       | KVPLFAWSVFITAWLILLSLPV                             | LAGAITMLLMDR <sup>249</sup>                                |
|    | E.coli bo3  | SPGVGVDYWTWSLOLSGTGTTLTG  | NFFVTTLKMRAPGMTME                        | KMPVFTWASLCANVLIIASFPI                             | LTVTVALLTLDR <sup>25</sup>                                 |
|    | Bo.taurus<br>B.subtilis caa3  | HAGASVDLTIFSLHLAGVSSILGAI<br>SKGHGIDFSILGLQISGLGTLIAGI  | INFLATTINMRAPGMTYN                       | IRLPLFTWTTFVASALILFAFPP                            | LTVGLALMMLDR <sup>222</sup>                                |
| A1 | M.tuberculosis  | SPGAGGDLWIMGLIVAGLGTILGA  | /NMITTVVCMRAPGMTME                       | RMPIFTWNIMVTSILILIAFPL                             | LTAALFGLAADR <sup>238</sup>                                |
|    | Ae.pernix 1   | TPGIGLDLAALAIFLFSLSVTLGTI<br>NYGLGTNLIQIALILSGLSSTLTGV  | INFLVTIAAMRAPGIGWE                       | KMPMFTWSILFTVILMLWAFPP                             | LMVGGALLLLDR <sup>223</sup>                                |
|    | S.acidocaldarius SoxM<br>H.halobium   | MSSPAVDMMMLGLHLTGVSATMGA  | INFTATTFTERGEDVGWE                       | PDLDIFSWTMLTOSGLILFAFPL                            | FGSALIMLLLDR <sup>200</sup>                                |
| Ē  | R.marinus caa3  | MTSSGVTWVALGVFILGFSSILTGI   | LNFIVTVHKMRAPGLTWS                       | RLPLFVWGLYATSIVQILATPV                             | LGITVLLLALER <sup>231</sup>                                |
|    | A.sp<br>Sy.sp   | TGQVGEGIWIISVLLLGTSSILGAI<br>SGKWGEELWILSLLLVGTSSILGAI  | INFLVTLLKMRIPSMGFF                       | IQMPLFCWAMFATSALVLLSTPV<br>ISMPLFCWAMLATSSLILLSTPV | LAAGLILLAFDL <sup>224</sup>                                |
|    | T.thermophilus caa3   | -SESCUDEVLAATLLLGESSLLGNA   | NEVATIYNDRAOGMSL                         | <i>IKMPIYVWSVFAASVLNLFSLAG</i>                     | LTAATLLVLLER <sup>223</sup>                                |
| A2 | D.radiodurans   | -NQTGVSVFMVAVILNGLGSLLGSA<br>NDAGVTALYVFVMHLLGASSIATAV  | ANFAATIVNLRAPGMGLW                       | VKMPVFAWSIFATSILQLLTLGG                            | LTAAALLTYLEI <sup>231</sup>                                |
|    | Aq.aeolicus Al<br>S.acidocaldarius SoxB   | NSOFHAFLEYTTPOLMGMAYLVMS  | TAVILOTAAFVILIADAY                       | ATKPKGERLNIFAAYGVAFSIV                             | IAVTLPALAAAT <sup>208</sup>                                |
| L  | Aq.aeolicus A2  | AHWTEYLGAVLLVLGSLVPFFI  | DWTPSATOWKRE-NPDC                        | KIPLAVFGTFVNFILWTIMIVP                             | VAIEILFOLLPL <sup>200</sup>                                |
|    | N.pharaonis ba3<br>T.thermophilus ba3   | AHPLFYLGLA-VFVVGTWLAGV  | /DWFTTWWSWKQD-NPGE<br>/TVLDLWRRWKAA-NPGE | CRIPLPTFMVLTTMIFWYLSSIG                            | VAASILLFLLPW<br>LVLEAVLFLLPW <sup>211</sup>                |
| в  | Ae permix 2   | ANPLEYTGAALLTVGSWVFM  | DIFRIVIKERRE-NPDI                        | KVPVATYGILTAWVIWLVATPP                             | VALEVLLLLVPM   |
|    | B.stearothermophilus ba(0)3   | AHAGFYIGLA-LVVVGSWVSGH<br>IPPYSEYVVSPLFFIGWLLLDAH   | FAMFAHYARWRK-AHRGQ                       | ASPLLTFMSVTNMALWLICTLG                             | VAATVVFQLIPW <sup>201</sup>                                |
| F  | A.ambivalens aa3<br>H.pylori cbb3   | WPLDTTW/WAW/UWG   | NMEGSMSVRRENT1                           | YVSLWYYIATYVGIAVMYIFNN                             | LSVPTYFVADMG <sup>196</sup>                                |
| c  | Br.japonicum cbb3   | GYLLGVTOSKEYAEPEWY  | DLWLTTVWVVYLLVFLA                        | TITKRKEPHIFVANWFYLAFIV                             | TIAVLHLGNNPA <sup>232</sup>                                |
|    | Rhd.capsulatus cbb3<br>Rh.meliloti cbb3   | GYLLGITQSREYAELNWHI   | DILVAVVWVAYLIAFLO                        | TIFKRKEPHIYVANWFYLSFIV                             | TIAMLHIVNNLA <sup>238</sup>                                |
| L  | Az.caudinolans cbb3   | GYLLGITQGKEYAEPEWY  | ADLWLTIVWVTYFLVFLC                       | TVLKRKEPHIYVANWFYLAFIL                             | TIAVLHLGNNAA <sup>253</sup>                                |
|    |   | Ø Ette  | VI                                       |  | VII  |
| Г  | P.denitrificans aa3   | NFGTQFFDPAGGGD-PVLYQHIL   | DD D K<br>FG <b>HPFW-</b> YIIILPGFGI     | K<br>ISHVISTFAKKPIFGYLPMV                          | LAMAAIG-ILGF <sup>320</sup>                                |
|    | E.coli bo3  | NFGTOFFDPAGGGD-PVLYQHIL<br>YLGTHFFTNDMGGN-MMMYINLIA<br>NLNTTFFDPAGGGD-PILYQHIFM<br>LFGTNFFNPELGGN-TVIWEHLFM<br>HLGAHIYDAANGGVLLWQHIFM | AWGHPEV-YILILPVFG                        | VFSEIAATFSRKRLFGYTSLV                              | WATVCIT-VLSF <sup>328</sup>                                |
|    | Bo.taurus   | NLNTTFFDPAGGGD-PILYQHLFW  | FFGHPEV-YILILPGFGN                       | ISHIVTYYS-GKKEPFGYMGMV                             | WAMMSIG-FLGF <sup>203</sup>                                |
| Al | B.subtilis caa3<br>M.tuberculosis   | LFGTNFFNPELGGN-TVIWEHLFW<br>HLGAHIYDAANGGVLLWOHLFW  | FGHPEV-YIIALPFFG                         | VSEIFPVFSRKPIFGYTTLV                               | YATLSIA-ALSV <sup>308</sup>                                |
|    | Ae.pernix 1   | NLGTEFFLNPAGGALLWDHLFW  | FFG <mark>HPEV-Y</mark> ILLFPALG         | MADVISTFSGKPIYAKRYIL                               | TAFLIAT-IISF <sup>295</sup>                                |
|    | S.acidocaldarius SoxM<br>H.halobium   | LWGTPFFDSALGGS-PVLWQQLFWI   | FFGUPEW-YILILPAMGI                       | JVSELLPKMARREIFGYTAIA                              | LSSIAIAFLSAL <sup>***</sup><br>VSTLAIG-VLSF <sup>338</sup> |
| -  | R.marinus caa3  | LWGTPFFDNPAGG-ALLWDALFM<br>LWGTPFFDSALGGS-PVLWQQLFM<br>NFGTTFFTVAGGD-PIFWQHLFM<br>IMKIGIFDPALGGD-PILFQHFFM                            | YSHPAV-YIMILPAFG                         | /ISELIGTFSRKGIFGYKFVA                              | LSSVAIA-FLGF <sup>302</sup>                                |
|    | A.sp  | IAGTTFFNPTGGGD-PVVYQHMFW<br>IAGTSFFNPVGGGD-PVVYQHLFW  | FYSHPAW-YIMILPFFGA                       | AISEIIPIHSRKPIFGYKAIA                              | YSSLAIS-FLGL <sup>303</sup>                                |
|    | Sy.sp<br>T.thermophilus caa3  | IAGTSFFNPVGGGD-PVVYQHLFW<br>KIGLSWFNPAVGGD-PVLFOOFFW  | YSEPAW-YIMILPFFG<br>YSEPTW-YVMLLPYLG     | LAEVASTFARKPLFGYRAIA                               | WAQMGIV-VLGT <sup>294</sup>                                |
| A2 | D.radiodurans   | KIGLSWFNPAVGGD-PVLFQQFFW<br>KLGLSMFNPGIGGV-PVLFQQFFW  | YSHPAW-YVMLLPYLG                         | GAEVASTMARKPLFGYRVMV                               | YSMLAIV-LVSC302  |
|    | Aq.aeolicus Al  | YLGTNFFNPAKGGD-PLIYQNIFW<br>LWYTLYFFANVPVN-NLLWAILFW  | •¥SBPVW-¥VOVLPVFGI                       | YSELLPVFARRPLFGITSMV                               | FAVWALT-AVSF   |
|    | <i>S.acidocaldarius</i> SoxB<br>Aq.aeolicus A2                                  | SLGLUDEINPLLARTLE   | FGPVV-YFWLLPAYVA                         | ALYTILPKIVSEKGKLYSDPAAR                            | LAFILFL-IFSL <sup>200</sup>                                |
| Ē  | N.pharaonis ba3   | SLGTVDOVNALLTRTLF   | FGAVW-FFWLMPAYM                          | WYIMLPKISGGKLFSDPLAR                               | VVFVLFL-VLST <sup>2</sup>                                  |
| в  | T.thermophilus ba3<br>Ae.pernix 2   | SFGLVEGVDPLVARTLFM<br>SLFGMEVDVLLARTLFM   | VFG PIN-YFWLLPAVAI                       | WYYAIPRILGVPLFSETMAK                               | VAYILYM-IAST <sup>204</sup>                                |
| B  | B.stearothermophilus ba(0)3   |   | VECTOT - VEWILLOAVM                      | WYAUT PRUTGCKMESDSLAR                              | LAFTLFL-LFST**   |
| Ļ  | A.ambivalens aa3  | IWDILAYAGIVGLN-PIANQIAFW<br>SVWHSISMYSGSNDALIQM   | IFGHAVV-YMAWLPAVA                        | LYLLIPTLANKPLYSDRMAR                               | ISALLYL-IFSN <sup>200</sup><br>FSFWSLM-FVVT <sup>264</sup> |
|    | H.pylori cbb3<br>Br.japonicum cbb3  | I. DUGAECSKSVVAWCCTODAMEO   | MYGHINANGEFLTAGELA1                      | MMYFTPKRAERPIYSYRLSI                               | THEWAPT-EPIT   |
| С  | Rhd.capsulatus cbb3   | VDVCTECTKCVALMACVADAMTA   | VC NAMOFFL TACELON                       | MYYFVPKOAERPVYSYKLSI                               | VHFWALI-FLYI   |
|    | Rh.meliloti cbb3<br>Az.caudinolans cbb3   | VPVSIIGIRSVQDMAGVQDALIQ<br>VPVSFLGSKSYSAFSGVQDALIQ<br>IPVSVFSPKSYIVWSGVQDAMVQ   | VYGINAWGFFLTAGFLAN                       | MYYFIPKQVNRPVYSYRLSI<br>MYYFIPKRADKPVYSYRLSI       | THFWALL-FMY1<br>VHFWALL-FLY1 <sup>326</sup>                |
| -  | Az.caudinorans coos   |   | Cu                                       |  |  |
|    |   | (Contraction) (Contraction)   | VIII<br>K K                              |  | <b>x</b> ()  |
| Г  | P.denitrificans aa3   | VVWA  | MTTAUPTCTRVFSWTA                         | MWGGSIEFKT   | PM <sup>373</sup>  |
|    | E.coli bo3  | IVWLIIIFFTMG-AGANVNAFFGIT<br>IVWAIIMFTVG-MDVDTRAYFTSA   | MITATPROVETENWLE                         | MYOGRIVEHS   | AM <sup>381</sup>  |
|    | Bo.taurus<br>B.subtilis caa3  | MOTOR MEMORY CONTANNATENVA  | MATATORCTRTENTALL                        |  | AM <sup>333</sup>  |
| A1 | M.tuberculosis  | ATTUA MEAN CAUTIDEECEM  | VITAUDUCTREENWIC                         | MWKCOLTEET   | PM <sup>359</sup>  |
|    | Ae.pernix 1   | VVWMHHMFITG-TNIYTRLFYSIT  | ILISIPFEMAVMSFIF                         | LYKGRLVYTV   | PM <sup>336</sup>  |
|    | S.acidocaldarius SoxM<br>H.halobium   | OTT IN THE FORMER TO DO TO CONVAL   |  |  |  |
| ٦  | R.marinus caa3  | GVWAHHMFTTG-IDFRIRSSFMAV<br>LVWGHHMFVSG-QSATAATVFSLL<br>IVWAHHMFTSG-IPGWLRMFFMIT  | FLIGVPTGVKVLNWVA                         | LYRGSIWLRT   | PL <sup>355</sup>  |
|    | A.sp<br>Sv.sp   | TIMIN MEMORY - TOCHT DMEEMAT  | MITAUDECTRIFENCE                         | ILWCCKTOLNS  | AM"  |
|    | <i>sy.sp</i><br>T.thermophilus caa3   | MUMUA MEMUC - FOUL FOLDER FR  | ALTAUD CURLENT IC                        | LWGGKLOMKT   | PL <sup>3*</sup>   |
| A2 | D.radiodurans   | IVWLHHMFAVG-IPEAWQIAFMIS<br>FLWIHHMFVSG-VPNWTRVLFSYT  | T.TVAVDTCVKTENI.TC                       | NT.WGGRTLMRM                                       | P1   |
|    | Aq.aeolicus A1<br>S.acidocaldarius SoxB   | FLWIHHMFVSG-VPNWTRVLFSYT<br>GVWVHHLQTWP-LPIVLREWVNLS<br>PVGLHHQFTDPGITNTWKLIHALF  | LILATGSGLTVLNLGL                         | IFTSRKYDWKD  | PV <sup>333</sup>  |
|    | Aq.aeolicus A2  | PVGL. QFTDPGITNTWKLIHALF  | FGVALPSMITAFTVAT                         | LEYSVKAEHPELKNSKFYWWTF                             | LPFMRLEGNKWM <sup>344</sup>                                |
| Г  | N.pharaonis ba3<br>T.thermophilus ba3   | PTGIHLQYLDPGIAEGFKFMAMVN<br>PVGFHLQFADPGIDPTWKMIHSVL  | I FVAVPSLMTAFTVAA                        | LEFAGRLRGGRGLF                                     | 'GWIRALPWDNPA'''   |
| в  |   | DVGI WOODVDDGTGDEEKI WUUU V   | TEXT A TO CMT TA ENTRA                   | T FDACDMDCCDCIV                                    | CWNFKLPWGSLA   |
|    | B.stearothermophilus ba(0)3   | PVGF <mark>HL</mark> QLLEPGISPFWKYVQVVL   | FMVIIPSLMTAFSMFA                         | FESYGRSQGAKGLF                                     | GWLRKLPWGDAR <sup>335</sup>                                |
| Ē  | Ae.pernix 2<br>B.stearothermophilus ba(o)3<br>A.ambivalens aa3<br>H.pylori cbb3 | NVPV LYMVN-LPVAIKVLQEIL<br>WAGGIILIYST-VPDWVOTLSSVF   | YAVVVPSMMTFLNLWA<br>SVVLILPSWGTAINMU     | ARGAQVNFNVI  |  |
|    | Br.japonicum cbb3   |   |  |  |  |
| С  | Rhd.capsulatus cbb3   | WAGPHILHYTA-LPDWASTLGMVM  | VILWMPSWGGMINGLM                         | LSGAWDKLRTD  | PV <sup>365</sup>  |
| Ľ  | Rh.meliloti cbb3<br>Az.caudinolans cbb3   | WAGP <b>HH</b> LHYTA-LPDWAQTLGMVF<br>WAGP <mark>HH</mark> LHYTA-LPDWAQTLGMTF  | SIMLWMPSWGGMINGLM                        | LSGAWDKLRTD  | PI <sup>380</sup>  |
|    |   | CuCu  |  |  |  |

Fig. 2 (continued).

|    | Г        | P.denitrificans aa3                             | LWAFGFLFLFT-VGGVTGVVLSQAPLDRVYHDUYYVVAHFHYVMSLGAVFGIFAGVYYWIGKMSGRQYPEWA444   |
|----|----------|---|---|
|    |          | E.coli bo3                                      | LWTIGFIVTFS-VGGMTGVLLAVPGARFVLHNSLFLIA  |
|    |          | Bo.taurus                                       | MWALGFIFLFT-VGGLTGIVLANSSLDIVLHDTYYVVAHFHYVLSMGAVFAIMGGFVHWFPLFSGYTLNDTW <sup>109</sup><br>LYAVSFIPSFV-LGGVTGVMLAAAAADYQFHDTYFVVAHFHYVIIGGVVFGLLAGVHFWWPKMFGKILHETM <sup>116</sup>      |
| A1 |          | B.subtilis caa3<br>M.tuberculosis               | LYAVSFIPSFV-LGGVTGVMLAAAAADYQFHDTYFVVAHFHVIIGGVFGLLAGVHFWPRHFGKIhheim<br>LFSVGFMVTFL-LGGLTGVLLASPPLDFHVTDSYFVVAHFHYVLFGTIVFATFAGIYFWFPRMTGRLLDERL <sup>430</sup>                        |
|    |          | Ae.pernix 1                                     | LFAVGALLNFI-IGGSTGVYLGSIAIDRGFRGTYWVVALIDYILVGTVTLGLIAGLYYWWPKITGRTYSERL <sup>419</sup>   |
|    |          | S.acidocaldarius SoxM                           | TLLISFTVMFL-LGGITGVFFPLVPIDYALNGTYFVVGHFYMVY-AILYALLGALFYYFPFWSGKWYNDDL <sup>406</sup>  |
|    | F        | H.halobium                                      | LFCIGFVQNFI-IGGVTGVFLAVIPIDLILHDTYYVVGHFFFIVYGAIGFALFAASYYWFPMVTGRMYQKRL <sup>462</sup>   |
|    |          | R.marinus caa3                                  | LYALAFLFVFP-IGGFTGIALGTLGLDVPLHDTYFVVAHFHYVMVSGGLLAFLGGLHYWWPKMFGRLYNEKL <sup>426</sup>   |
|    |          | A.sp<br>Sy.sp                                   | LFAAGFVGTFV-IGGVSGVMLAAVPFDIHVHDIYFVVAHU YVLFGGSVLGIFAAIYHWFPKMTGRMINEFW <sup>427</sup><br>LFAFGFLSSFM-IGGLTGVMVASVPFDIHVHDIYFVVGHFHYVLFGGSAFALFSGVYHWFPKMTGRMVNEPL <sup>419</sup>      |
| A2 |          | T.thermophilus caa3                             | YWVLGFTFNFL-LGGTTGVMLSMTPLDYOFHDSYFVYA  |
| A2 |          | D.radiodurans                                   | YWLIGFIFNFL-IGGITGVSLGMIPFDYQVTMSYYVVAHFENVMMFGTAFLAMAGLYYWWPKMTGRFLDEKV <sup>426</sup>   |
|    |          | Aq.aeolicus Al<br>S.acidocaldarius SoxB         | $\label{eq:light} LYALGGVFMFL-IGGLTGLPLGMVAIDLGVSD CFVVGHFH YVLGMAITLGILGAVIHWFPLATGKYYPENL^{364} GMGALISLIGFILAGAQALVLPENSINPLFHNSYYVVGHFHLMIWTLIIMGYTTVFLDMLRTSFAGFNFSATSSKW^{109}$   |
|    |          | Ag.aeolicus A2                                  | FSYFFAGLVLFFTGGTTGTVNASYNVNLVVHNAYVPGHIPTTVGGLVLLVFFALSLYMVSKLRGSEVKLKGL <sup>417</sup>   |
|    | Ē        | N.pharaonis ba3                                 | FTGMALAGIMFAAAAFSGMVNAGMNINYLVHNNWVVGHIPLTVGTAVALTFMAVSYWFLPOITGKKLWGKSV <sup>413</sup>   |
| P  |          | T.thermophilus ba3                              | FVAPVLGLLGFIPGGAGGIVNASFTLDYVVHNTAWVPGHFHLQVASLVTLTAMGSLYWLLPNLTGKP-ISDAQRRL <sup>420</sup>   |
| в  |          | Ae.pernix 2                                     | FSGLMLPIILFANGGITGIINASFQLMTVVHNUTWIVGHFHTTVGGASALTFMAAMLLLAREMMKREVIAPWL <sup>405</sup><br>FFAPFVGMLFFIPAGTGGIINASHQLNQVVHNUIWVTGHFHLTVATTVVLTFFGASYWLIPHLTGR-VLTKAMNRL <sup>410</sup> |
|    | L        | A.ambivalens aa3                                | FFAPFVGMLFF1PAGIGGTINASHQLQUVHNITWVIGHDINATIVVIITFGASHWITHINGK VIIAMAKU<br>FVATSFAGAIAAGVTGIANATIAFDSIVHNSMWVVGHDAMILLSIVPAAMAVLYFMIPMMTGRQWYSSKM <sup>413</sup>                        |
|    | Г        | H.pylori cbb3                                   | TKFLVLASTFYMLSTLEGSIOAIKSVNALAHFNDWIIGWVHDGVLGWVGFTLIASMYHMTPRLFKREIYSGRL <sup>391</sup>  |
| С  |          | Br.japonicum cbb3                               | LRMLAX/SVAFYGMSTFEGPMMSIKVVNSLSHYNDWTIGNVNSGALGWVGFVSFGALYCLVPWAWNRKGLYSLKL453  |
| C  |          | Rhd.capsulatus cbb3                             | IRMMVVSIGFYGMSTFEGPMMSIKAVNSLSHYTDWTIGHVHSGALGWNGMITFGMLYFLTPRLWGRSGLYSLKL <sup>437</sup>   |
|    | L        | Rh.meliloti cbb3<br>Az.caudinolans cbb3         | VRMMVMAVAFYGMATFEGPMMSIKTVMSLSHYTDWTIGHVHSGALGWNGLITFGAIYYLVPKLWNRERLYSVRM <sup>439</sup><br>IRMMVVAVAFYGMATFEGPMMSVKSVMSLSHYTEWGIGHVHSGALGWVAYISFGAIYCLIPWLWNKREMYSMKA <sup>454</sup>  |
|    |          | Az. caudinorans cobs                            |   |
|    |          |   | (XI )   |
|    | Г        | P.denitrificans aa3                             | GQLHFWMMFIGSNLIFFPQHFLGRQ-GMPRRYIDYPVEFAYWNNISSIGAYISFASFLFFIGIVF <sup>508</sup>  |
|    |          | E.coli bo3                                      | GKRAFWFWIIGFFVAFMPLYALGFM-GMTRRLSQQIDPQFHTMLMIAASGAVLIALGILCLVIQMY <sup>517</sup><br>AKIHFAIMFVGVNMTFFPQHFLGLS-GMPRRYSDYPDAYTMWNTISSMGSFISLTAVMLMVFIIW <sup>473</sup>                   |
|    |          | Bo.taurus<br>B.subtilis caa3                    | GKISFVLFFIGFHLTFFIQHFVGLM-GMPRRVYTFLPGQLETGNLISTIGAFFMAARVILLLVNVI  |
| A1 |          | M.tuberculosis                                  | CKLHFWLTFIGFHTTFLVOHWLGDE-GMPREYADYLPTDGFOGLNVVSTIGAFILGASMFPFVWNVF***  |
|    |          | Ae.pernix 1                                     | GKIHFALAMLGVALTFLPQFALMDMPRRYFTYDIPEWVPLNQLSTLGAFIFGGSMAIGLVNFL <sup>482</sup>  |
|    |          | S.acidocaldarius SoxM                           | GKTGAILLVAGTFLTATGMSIAGIL-GMPRRYAVIPSPIVDPFQFMASVGAVLTGIGLFILAGVLV <sup>471</sup><br>AHAHFWTALVGSNATFLAMLWLGYG-GMPRRYATYIPQFATAHRLATVGAFLIGVSTLIWLFNMA <sup>526</sup>                   |
|    | F        | H.halobium<br>R.marinus caa3                    | AOTAALLIFVGFNVTFFPOFILGTO-GMPRRYFDYVPEFTTLHQLSTVGSWILGLGLLLVAVCLL***  |
|    |          | A.sp  | GKVHFALTIVGLNMTFLPMHKLGLM-GMNR IAOYDPKFTLLNEICTYGSYILAVSTFPFIFNAI   |
|    |          | Sy.sp   | GRLHFILTFIGMNLTFMPMHELGLM-GMNRTIALYDVEFQPLNVLSTIGAYVLAASTIPFVINVF <sup>483</sup>  |
| A2 |          | T.thermophilus caa3                             | GRLHFWLFLVGYLLTFLPQYALGYL-GMPRRYYTYNADIAGWPELNLLSTIGAYILGLGGLVWIYTMW <sup>485</sup><br>GLAHFWLFMIGSWLTFLPQYILGLL-GMPRRYYTYPAGNWAWTELNFASTVGAFLLLLGGLAMLYNMF <sup>493</sup>              |
|    |          | D.radiodurans<br>Aq.aeolicus A1                 | CKLSFWLTFAGANTFYFLOWVAGML-GNPREYPDYPPIPEWTTLHVIOTIGALILGVGVVIFTYGII   |
|    |          | S.acidocaldarius SoxB                           | MRTGMTWWTAPFMGVGYAMSVAGYL-GFLR MIAYPVIFOPYNLVESFLAEIGIPGLLLTLFVGM*'   |
|    | Ľ        | Aq.aeolicus A2                                  | AVI.APYFWMOGMFMFSYAMMVGGVVVGFPRRTNAGLTYLNPDSPLYRPEWTGYAOLAAVGGVLLAIGFAFYFASLI   |
|    |          | N.pharaonis ba3<br>T.thermophilus ba3           | ALAQVVLWFVCMTFMSNAMHRSGLA-GMPRTAEPQYRNFEFEMAAG-SLGELNAQVVLGGILLFVSTLLFVLVVV <sup>487</sup><br>GLAVVWLWFLGMMIMAVGLHWAGLL-NVPRAYIAQVPDAYPHAAVPMVFNVLAGIVLLVALLLF1 <sup>485</sup>          |
| в  |          | Ae nernix ?                                     | AVAAFYMWTVGOYLESTGYYVAGVM-GAPRETSELLYGGLAPTEWIPWLOLGAIGGMVFWIAGVILVGLIA   |
|    |          | B.stearothermophilus ba(0)3                     | ATTOTTWAVGMTFMSGSMHFAGLL-GAPRESAFSTYGNSPOALEWIPYOIAOAVGGTILFIGIILMLVIVI   |
|    | F        | A.ambivalens aa3                                | AWIHYIGYTIGASILIIGFEMIGFY-GVVRAEIYPRVPGLVFAENLATAGALIAEIATLVWFVNLV <sup>479</sup><br>VDFQFWIMTLGIVLYFSSMWIAGITQGMMWRDVDQYGNLTYQFIDTVKVLIPYYNIRGVGGLMY <sup>455</sup>                    |
|    |          | H.pylori cbb3<br>Br.japonicum cbb3              | VDFQFWIMTEGIVEYSSMWIAGITQGMMWDVDQTGNEITQFEDIVKVHFFINIKGVGGEMI<br>VNWHFWVATLGIVEYISAMWVSGILQGLMWRAYTSLGFLEYSFIETVEAMHPFYIIRAAGGGEF <sup>517</sup>  |
| С  |          | Rhd.capsulatus cbb3                             | VSWHFWLATIGIVLYASAMWVTGIMEGLMWREVDAOGFLVNAFADTVAAKFPMYVVRGVGGVLY <sup>301</sup>   |
|    |          | Rh.meliloti cbb3                                | VNWHFWLATLGIVVYAAVMWVAGTOOGLMWREYDDOGFLVYSFAETVAAMFPYYVMRAAGGALF <sup>303</sup>   |
|    | L        | Az.caudinolans cbb3                             | IEWHFWVSTLGIVLYICAMWVAGILQGLMWRAYTALGFLEYSFIETVEAMHPLYVIRAIGGILF <sup>518</sup>   |
|    |          |   |   |
|    | <b>–</b> | P.denitrificans aa3                             | YTLFAGKRVNVPNYWNEHADTLEWTLP-SPPPEHTFETLPKREDWDRAHAH558  |
|    |          | E.coli bo3                                      | VSIRDRDONRDLTGDPWGGRTLEWATS-SPPPFYNFAVVPHVHERDAFWEMKEKGEAYKKPDHYEEIHMPKNS <sup>589</sup>  |
|    |          | Bo.taurus                                       | EAFASKREVLTVDLTTTNLEWLNG-CPPPYHTFEEPTYVNLK  |
| A1 |          | B.subtilis caa3                                 | WTSVKGEYVGADPWHDGRTLEWTVS-SPPPEYNFKQLPFVRGLDPLWIEKQAGHKSMTPAEPVDDIHMPNG <sup>552</sup><br>KSWRYGEVVTVDDPWGYGNSLEWATS-CPPPRHNFTELPRIRSERPAFELHYPHMVERLRAEAHVGRHHDEP <sup>567</sup>       |
| Л  |          | M.tuberculosis<br>Ae.pernix 1                   | YSLVKGGSAAPNPWNSWTLEWFTN-SPPPKHNFDGVPVVRKDNTVVFVSEEALSKYGKDAIVEGRVDVSN <sup>231</sup>   |
|    |          | S.acidocaldarius SoxM                           | HGVFRGRAVNGVDPWDNTSVKLODFFTKPVKLPLSFGKSLDGAFDEEYHGIKFPYYSVLGLFLSFIPLGFM <sup>542</sup>  |
|    | L        | H.halobium                                      | TSWREGPRVDSTDPWDLEETDQFTNDWAWFRAKEETTVLPDGGDEAQSEADAVTDGGQPAADSDTES <sup>593</sup>  |
|    |          | R.marinus caa3                                  | $\label{eq:hsllkgqqapanpwgagtlewthtgrlpsphnfertpvvtrgpvdyhlaeeifgnghgdgsstpavipaq^{56} \\ wswlygekagnnpwraltlewmtt-sppaienfdklpvlatgpvdyglekasegvplsdpnpvlsagpns^{560} \\ \end{array}$  |
|    |          | A.sp<br>Sv.sp                                   | WSLFKGEKAARNPWRALTLEWOTA-SPPIIENFEEEPVLWCGPYDFGIDTELMDDEETVOTLIADAAGS-331   |
|    |          | T.thermophilus caa3                             | KSLRSGPKAPDNPWGGYTLEWLTA-SPPKAHNFDVKLPTEFPSERPLYDWKKKGVELKPEDPAHIHLPNS <sup>334</sup>   |
| A2 |          | D.radiodurans                                   | OSEKRETTAGENEWGGETLEWTSS-SPEAAYNFAHDFPONFPTERPLYDWEONGETLTPVDPKSIHLPQD <sup>302</sup>   |
|    |          | Aq.aeolicus Al                                  | KGLKSGEKASDNPWKSPSLEWKVG-VPARAHGHGDHPPRVEPDWHPYRYDKEEILK485<br>FDALRYASKQPVFSSPSVSSFSMQVDKGELVKKIDSEKGVNNVG   |
|    |          | <i>S.acidocaldarius</i> SoxB<br>Ag.aeolicus A2  | atal a prure stiller pradayhda pa pli NNI ktwtva a i i avlsy i pplyda svrgvFFKSPAYNEKFPMPLKO <sup>303</sup>   |
|    | F        | N.pharaonis ba3                                 | MTVLGDKAEPGTLPAN-EYADTLSGPED-SPKVLDNLKLWTAIAVILVILAYALPLASIISRGGVFGPGVGTYP <sup>339</sup>   |
|    |          | T.thermophilus ba3                              | YGLESVLLSRERKPELAEAPLPFAEVISGPEDRRLVLAMDRIGFWFAVAAILVVLAYGPTLVQLFGHLNPVP <sup>337</sup>   |
| в  | 1        | Ae.pernix 2                                     | ATLLFGKKVSVPQGERVLAFDET-TPGRIENGKYILEEVRFWIVVTIALAILGYIGPLYEIFARGLVPV <sup>543</sup><br>NLAFAPKGETEFPVAEAAAPQERVVLVFENWKLWIGIVVALILIAYTVPLIDIIQNAPPGSK <sup>544</sup>                   |
|    | L        | B.stearothermophilus ba(0)3<br>A.ambivalens aa3 | ATLVKGRTARLEGI.SLGOLINTVAMSLDWSSAGFKLNNFNKTGKTMNSKALGAYWALGIIGALIIVISTIPLALGG   |
|    | Ē        | H.pylori cbb3                                   | FTGFTTFAVNTFMTTTAGKKIEREPNVATPMSR   |
| с  | 1        | Br.japonicum cbb3                               | LTGALTMAYNLWMTVRVGEAEVOMPVALOPAE  |
| C  | 1        | Rhd.capsulatus cbb3<br>Rh.meliloti cbb3         | LIGGLIMAYNLWATVAKQEKTANLAVAVPAE   |
|    | L        | Az.caudinolans cbb3                             | LAGSLIMARNVFMTITRAETVSQPSGAALAPAE51   |
|    |          |   |   |

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*<sub>3</sub>, P24010, *stearothermophilus ba*<sub>3</sub>, 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*<sub>3</sub>, P98005, *ba*<sub>3</sub>, 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<sub>B</sub> are indicated by a, a<sub>3</sub> and Cu, respectively. Transmembrane helices for the *P. denitrificans aa*<sub>3</sub> 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*<sub>3</sub> 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 CuA centre, as this loss could be easily reverted by mutating a few residues, as it was done for the *Escherichia coli bo*<sub>3</sub> 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<sub>A</sub> 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<sup>1</sup>) are strictly conserved in all haem-copper oxidases (Fig. 2). The prokaryotic oxidases may contain quite diverse haems: A, B, O and derivatives

<sup>&</sup>lt;sup>1</sup> 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 <sub>2</sub> -channel                                |
| Tryptophan (TrpI-272)                 | $\pi$ interaction with HisI-326                        |
| Arginine (ArgI-474)                   | H-bond to $\delta$ -propionate of the low-spin haem    |
| Arginine (ArgI-54) <sup>a</sup>       | 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) <sup>b</sup> | Possible electron transfer between the haems           |
| Threonine/serine (ThrI-361)           | Unknown  |
| Threonine/serine (ThrI-405)           | Unknown  |

<sup>a</sup>Only in haem *a* low spin-containing oxidases.

<sup>b</sup>Absent in *cbb*<sub>3</sub> 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 bo3 oxidase from E. coli, and a significant increase in  $K_{\rm M}$  for oxygen was observed, without any change in  $V_{\text{max}}$  [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  $\pi$  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  $\delta$ -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

| Domain   | Subdomain/group/order/genus    | Oxidase                                 | Chanr          | nel residues |                  |     |     |   |
|----------|--------------------------------|---|----------------|--------------|------------------|-----|-----|---|
|          |                                |   | 'D'a           |              | 'K' <sup>b</sup> |     |     |   |
|          |                                |   | E/Y            | Others       | K/T              | T/S | S/Y | Y |
| Bacteria | Purple bacteria                | aa <sub>3</sub>                         | Е              | +            | K                | Т   | S   | Y |
|          |                                | caa <sub>3</sub>                        | Е              | +            | Κ                | Т   | S   | Y |
|          |                                | $bo_3$                                  | E              | +            | Κ                | Т   | S   | Y |
|          |                                | $bb_3$ ( $ba_3$ )                       | Е              | +            | Κ                | Т   | S   | Y |
|          | Gram-positive                  | <i>aa</i> <sub>3</sub>                  | Е              | +            | Κ                | Т   | S   | Y |
|          | -                              | caa <sub>3</sub>                        | Е              | +            | Κ                | Т   | S   | Y |
|          |                                | ba <sub>3</sub> (B. stearothermophilus) | Y <sup>c</sup> | _            | Т                | S   | Y   | Y |
|          | Cyanobacteria                  | <i>aa</i> <sub>3</sub>                  | Y              | +            | Κ                | Т   | S   | Y |
|          | Flexibacter, Bacteroides and   | caa <sub>3</sub>                        | Y              | +            | K                | Т   | S   | Y |
|          | Cytophaga (R. marinus)         |   |                |              |                  |     |     |   |
|          | Thermus and Deinococcus        | caa <sub>3</sub>                        | Y              | +            | K                | Т   | S   | Y |
|          |                                | $ba_3$                                  | T(?)           | _            | Т                | S   | Y   | Y |
|          | Aquificiales                   | Aquifex aeolicus A1 <sup>d</sup>        | Y              | +            | K                | Т   | S   | Y |
|          |                                | A2 <sup>d</sup>                         | _              | —            | Т                | S   | Y   | Y |
|          | Purple bacteria and R. marinus | $cbb_3$                                 | Y              | —            | _                | S   | Y   | _ |
| Archaea  | Crenarchaeota                  | SoxB (S. acidocaldarius)                | Y              | —            | Т                | S   | Y   | Y |
|          |                                | SoxM (S. acidocaldarius)                | Е              | +            | K                | S   | S   | Y |
|          |                                | Aeropyrum pernix 1 <sup>d</sup>         | Е              | +            | —                | _   | —   | Y |
|          |                                | Aeropyrum pernix 2 <sup>d</sup>         | —              | _            | Т                | S   | Y   | Y |
|          |                                | aa <sub>3</sub> (A. ambivalens)         | _              | _            | Т                | S   | Y   | Y |
|          |                                | Pyrobaculum aerophilum <sup>d</sup>     | Е              | +            | Κ                | S   | S   | Y |
|          | Euryarchaeota                  | ba <sub>3</sub> (N. pharaonis)          | Y <sup>c</sup> | _            | Т                | S   | Y   | Y |
|          | -                              | Halobacterium halobium <sup>d</sup>     | Е              | +            | Κ                | S   | S   | Y |
| Eukarya  |                                | <i>aa</i> <sub>3</sub>                  | Е              | +            | Κ                | Т   | S   | Y |

Table 2 Proton channels in haem-copper oxidases

<sup>a</sup>GluI-278, TyrI-256 (R. marinus); Others: AspI-124, AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134 and SerI-193.

<sup>b</sup>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). <sup>c</sup>This tyrosine is one residue before in the pattern –YSHPXV–.

<sup>d</sup>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<sub>B</sub>. 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 aa3 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 cbb3 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 A [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<sub>B</sub> (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*<sub>3</sub> oxidases from the prokaryotes *Rhodothermus marinus* [42], *T. thermophilus* [43] and *Deinococcus radiodurans* [44], oxidase 1 from *Aquifex aeolicus* 

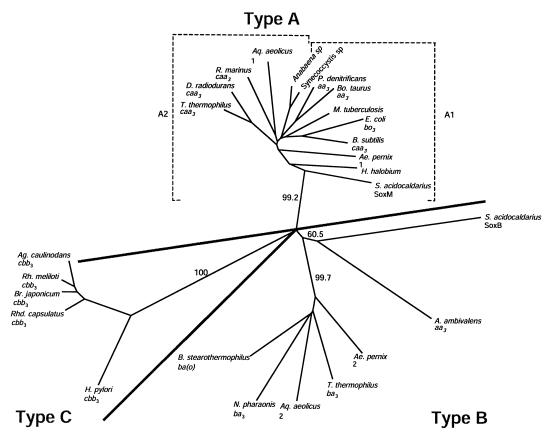


Fig. 3. Phylogenetic tree for the haem-copper oxidase subunit I, based on the amino acid sequence alignment of Fig. 2. The tree was made using Clustal W Version 1.6 [90] excluding positions with gaps and correcting for multiple substitutions. Bootstrap values for the main branches are indicated.

[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<sup>274</sup>GHPEV<sup>279</sup>- (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  $C_{\beta}$ - $C_{\gamma}$  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<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> 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

| Oxi | dase type                                    | Subu | init I |     |                  |     |   | Subunit | II/FixP <sup>c</sup> |                      |         |        |         |
|-----|--|------|--------|-----|------------------|-----|---|---------|----------------------|----------------------|---------|--------|---------|
|     |  | Chai | nnels  |     |                  |     |   | Haem I  | Haem II              | Cupredoxin<br>domain | Helices | Loop I | Loop II |
|     |  | 'D'a |        |     | 'K' <sup>b</sup> |     |   |         |                      |                      |         |        |         |
|     |  | E/Y  | Others | K/T | T/S              | S/Y | Y |         |                      |                      |         |        |         |
| A1  | (e.g., cyt oxidase <i>caa</i> <sub>3</sub> ) | Е    | +      | K   | Т                | S   | Y | _       | +                    | +                    | 2       | _      | _       |
|     | (e.g., cyt oxidase <i>aa</i> <sub>3</sub> )  |      |        |     |                  |     |   | _       | _                    | +                    | 2       | _      | +       |
|     | (e.g., quinol oxidase bo <sub>3</sub> )      |      |        |     |                  |     |   | _       | —                    | +                    | 2       | _      | —       |
| A2  | (e.g., cyt oxidase <i>caa</i> <sub>3</sub> ) | Y    | +      | Κ   | Т                | S   | Y | _       | +                    | +                    | 2       | _      | —       |
|     | (e.g., cyt oxidase <i>aa</i> <sub>3</sub> )  |      |        |     |                  |     |   | _       | —                    | +                    | 2       | +      | —       |
| В   | (cyt/quinol oxidases)                        | _    | _      | Т   | S                | Y   | Y | _       | _                    | +                    | 1       | +      | _       |
| С   | (cyt oxidase)                                | Y    | _      | _   | S                | Y   | _ | +       | +                    | _                    | 1       | _      | _       |

<sup>a</sup>GluI-278, TyrI-256 (*R. marinus*); Others: AspI-124, AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134 and SerI-193.

<sup>b</sup>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). <sup>c</sup>Dihaemic subunit of type C oxidases. and aa3 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<sub>B</sub> (TyrI-280). An equivalent for the Dchannel has yet to be fully established. The crystallographic structure of the ba<sub>3</sub> 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 aa3 oxidase (PheI-274Thr, GluI-278Ala) shows that ThrI-156 of T. thermophilus ba<sub>3</sub> oxidase may indeed be part of a proton channel [57].

A third family of haem-copper oxidases is composed of the *cbb*<sub>3</sub> 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<sup>2</sup>.

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<sup>+</sup>/e [58–60]. Type A2 enzymes also pump protons, with the same stoichiometry [48,61], emphasising the

<sup>&</sup>lt;sup>2</sup> 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) |

| Туре     | A1             |          |              |            |               |           |           | Туре      | A2       |          |                    |           | Туре         | В          |              |                |                |                |              |           | Туре                 | С            |            |                      |            |
|----------|----------------|----------|--------------|------------|---------------|-----------|-----------|-----------|----------|----------|--------------------|-----------|--------------|------------|--------------|----------------|----------------|----------------|--------------|-----------|----------------------|--------------|------------|----------------------|------------|
| P. den   | E. coli        | Bo.      | В.           | М.         | Ae.           | S. acid   | Н.        | R. mar    | А.       | Sy.      | T. the             | rD.       | Aq.          | S. acid    | Aq.          | N. pha         | r T. ther      | Ae.            | B. stear     | A. amb    | Br.                  | Н.           | Rhd.       | Rh.                  | Ag.        |
| aa3<br>1 | <sup>bo3</sup> | tau<br>3 | sub aa3<br>4 | tuber<br>5 | pernix 1<br>6 | SoxM<br>7 | halo<br>8 | caa3<br>9 | sp<br>10 | sp<br>11 | <i>саа</i> 3<br>12 | rad<br>13 | aeol 1<br>14 | SoxB<br>15 | aeol 2<br>16 | <sup>ba3</sup> | <sup>ba3</sup> | pernix 2<br>19 | ba(o)3<br>20 | aa3<br>21 | <sup>jap</sup><br>22 | pylori<br>23 | caps<br>24 | <sup>mel</sup><br>25 | caul<br>26 |
|          | 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         |
|          |                | 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         |
|          |                |          | 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         |
|          |                |          |              | 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         |
|          |                |          |              |            | 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         |
|          |                |          |              |            |               | 34        | 38        | 36        | 36       | 37       | 36                 | 37        | 33           | 18         | 20           | 19             | 25             | 23             | 21           | 18        | 14                   | 14           | 13         | 14                   | 12         |
|          |                |          |              |            |               | 59        | 59        | 60<br>26  | 60       | 58       | 61                 | 60<br>25  | 58           | 38         | 38           | 40             | 46             | 41             | 39           | 38        | 30                   | 35           | 31         | 31                   | 30         |
|          |                |          |              |            |               |           | 37        | 36        | 35       | 37       | 35                 | 35        | 33           | 21         | 22           | 20             | 24             | 22             | 21           | 18        | 13                   | 15           | 14         | 16                   | 1.         |
|          |                |          |              |            |               |           | 60        | 59<br>27  | 60       | 60       | 58                 | 57        | 57           | 39<br>20   | 42           | 42             | 44             | 43             | 41           | 37        | 31                   | 34           | 31         | 31                   | 31         |
|          |                |          |              |            |               |           |           | 37<br>63  | 44<br>68 | 45<br>68 | 39<br>61           | 36<br>57  | 34<br>56     | 20<br>36   | 23<br>41     | 23<br>44       | 22<br>43       | 22<br>41       | 22<br>44     | 20<br>39  | 15<br>32             | 17<br>35     | 16<br>33   | 16<br>33             | 15<br>33   |
|          |                |          |              |            |               |           |           | 03        | 45       | 45       | 40                 | 37<br>39  | 30<br>40     | 30<br>17   | 21           | 44<br>20       | 43<br>22       | 21             | 44<br>18     | 39<br>18  | 32<br>13             | 33<br>14     | 35<br>15   | 33<br>14             | 12         |
|          |                |          |              |            |               |           |           |           | 43<br>68 | 43<br>68 | 40<br>66           | 59<br>62  | 40<br>61     | 36         | 41           | 20<br>42       | 43             | 41             | 41           | 18<br>37  | 15<br>32             | 36           | 15<br>36   | 34                   | 33         |
|          |                |          |              |            |               |           |           |           | 00       | 76       | 41                 | 41        | 42           | 17         | 19           | 42<br>19       | 18             | 19             | 19           | 19        | 14                   | 16           | 16         | 16                   | 1:         |
|          |                |          |              |            |               |           |           |           |          | 89       | 65                 | 64        |              | 39         | 39           | 41             | 40             | 39             | 40           | 38        | 32                   | 36           | 34         | 34                   | 33         |
|          |                |          |              |            |               |           |           |           |          | 0)       | 43                 | 42        | 40           | 19         | 20           | 19             | 22             | 20             | 21           | 20        | 16                   | 17           | 16         | 16                   | 10         |
|          |                |          |              |            |               |           |           |           |          |          | 67                 | 65        | 59           | 38         | 39           | 42             | 42             | 40             | 42           | 40        | 34                   | 37           | 35         | 35                   | 35         |
|          |                |          |              |            |               |           |           |           |          |          | 07                 | 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         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           | 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         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              | 20         | 20           | 18             | 21             | 23             | 17           | 18        | 14                   | 16           | 14         | 13                   | 12         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              | 39         | 37           | 38             | 38             | 42             | 38           | 36        | 32                   | 36           | 31         | 31                   | 32         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            | 20           | 18             | 19             | 19             | 18           | 22        | 13                   | 15           | 16         | 14                   | 14         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            | 36           | 35             | 38             | 35             | 37           | 38        | 31                   | 35           | 32         | 31                   | 31         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            |              | 39             | 41             | 45             | 42           | 20        | 14                   | 15           | 12         | 13                   | 13         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            |              | 58             | 60             | 64             | 62           | 40        | 31                   | 34           | 30         | 29                   | 29         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            |              |                | 43             | 38             | 44           | 20        | 14                   | 16           | 15         | 15                   | 15         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            |              |                | 60             | 63             | 67           | 42        | 33                   | 37           | 33         | 32                   | 33         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            |              |                |                | 38             | 45           | 24        | 15                   | 18           | 16         | 16                   | 16         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            |              |                |                | 61             | 67           | 40        | 32                   | 35           | 33         | 32                   | 32         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            |              |                |                |                | 41           | 21        | 15                   | 18           | 14         | 15                   | 16         |
|          |                |          |              |            |               |           |           |           |          |          |                    |           |              |            |              |                |                |                | 65           | 43        | 35                   | 37           | 32         | 34                   | 33         |

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| Type A1 | A1                 |     |         |       |   |         |      | Type A2 | A2 |     |            |     | Type B | В       |        |         |                     |          |           |                 | Typ   | Type C |      |     |       |
|---------|--------------------|-----|---------|-------|---|---------|------|---------|----|-----|------------|-----|--------|---------|--------|---------|---------------------|----------|-----------|-----------------|-------|--------|------|-----|-------|
| P. den  | P. den E. coli Bo. | Bo. | В.      | М.    | Ae.                                     | S. acid | H.   | R mar   | A. | Sy. | T. ther D. | D.  | Aq.    | S. acid | Aq.    | N. phar | N. phar T. ther Ae. | Ae.      | B. stea   | B. stear A. amb | b Br. | H.     | Rhd. | Rh. | Ag.   |
| aa3     | $bo_3$             | tau | sub aa: | tuber | sub aa <sub>3</sub> tuber pernix 1 SoxM | SoxM    | halo | caa3    | ds | ds  | caa3       | rad | aeol 1 | SoxB    | aeol 2 | $ba_3$  | $ba_3$              | pernix 2 | $ba(o)_3$ | $aa_3$          | jap   | pylori |      | ləm | cauld |
| 1 2 3   | 7                  | б   | 4       | 5     | 9                                       | 7       | 8    | 6       | 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   | 09  | 59    |
| 24      |                    |     |         |       |   |         |      |         |    |     |            |     |        |         |        |         |                     |          |           |                 |       |        |      | LL  | 70    |
|         |                    |     |         |       |   |         |      |         |    |     |            |     |        |         |        |         |                     |          |           |                 |       |        |      | 90  | 84    |
| 25      |                    |     |         |       |   |         |      |         |    |     |            |     |        |         |        |         |                     |          |           |                 |       |        |      |     | 76    |
|         |                    |     |         |       |   |         |      |         |    |     |            |     |        |         |        |         |                     |          |           |                 |       |        |      |     | 87    |
| 26      |                    |     |         |       |   |         |      |         |    |     |            |     |        |         |        |         |                     |          |           |                 |       |        |      |     |       |

Table 4 (continued).

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<sub>3</sub> quinol oxidase [56]. While for the first two enzymes a stoichiometry of ca. 0.5H<sup>+</sup>/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 aa3 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<sup>+</sup>/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<sup>+</sup>/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*<sub>3</sub> 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-

| <u>^</u> | □ P.denitrificans aa3                             | QUVLGDLPVIGKPVNGGMNFQPASSPLAHDQQWL  | 34        |
|----------|---|---|-----------|
|          | Bo.taurus   | MAYPMQLGFQDATSPIMEEL  | 20        |
|          | Ae.pernix 1                                       | MDVIPTEAIWWRL   | 13<br>43  |
| A1       | E.coli bo3  | MRLRKYNKSLGWLSLFAGTVLLSGCNSALLDPKGQIGLEQRSL   |           |
|          | B.subtilis caa3                                   | MVKHWRLILLLALVPLLLSGCGKPFLSTLKPAGEVADKQYDL  |           |
|          | M.tuberculosis                                    | MIQEAPTVTPRGPGRLQRLSQCRPQRGSGGPARGLRQLALAAMLGALAVTVSGCSWSEALGIGWPEGITPEAHLNREL  |           |
|          | S.acidocaldarius SoxH<br>R.marinus caa3           | MSGMILLQNTAWLPEAASSIAPEVDSL   | 27        |
|          | T.thermophilus caa3                               | MORSFAALGLWGLSLAQEAHRVAITHPGGSFNQEVAFL  | 38        |
| A2       | Sy.sp   | VKIPGSVITLLIGVVITVVSLWYGQNHGLMPVAASADAEKVDGI  | 44        |
|          | A.sp  | MKIPSSIWTLLIGIGLTLASLWYGQNHGLMPVAASDEADLVDGL  | 44        |
|          | Aq. aeolicus B1                                   | MEQVIALPYKWWAEA   | 15        |
|          | N.pharaonis ba3                                   |   |           |
|          | T.thermophilus ba3                                |   |           |
|          | B.staerothermophilus ba3<br>S.acidocaldarius SoxA |   |           |
| В        | Ag.aeolicus B2                                    |   |           |
|          | A.ambivalens                                      |   |           |
|          | Ae.pernix 2                                       |   |           |
|          |   |   |           |
|          |   |   | 109       |
|          | P.denitrificans aa3                               | DHFVLYIITAVTIFVCLLLLICIVRFNRRANPVPARFTHNTPIVIWTLVPVLILVAIGAFSLPILFRSQEMPND<br>LHFHDHTLMIVFLISSLVLYIISLMLTTKLTHTSTMDPQEVETIWTILPAIILILIALPSLRILYMMDEINNP   | 93        |
|          | Bo.taurus<br>Ae.pernix 1                          | LHFHDHTLMIVFLISSLVLYIISLMLFTALTHISIMDFQLVm11FATIHIHIAFSBRITTMMDFINN<br>FLLFTAVGVLAAGTVTAFFIYSLFKYRSSGQALGEDQGTAGRIYRIMVESPVSGKSKYLLFVTGIIVMGLIVATI  | 88        |
| A1       | E.coli bo3  | ILTAFGLMLIVVIPAILMAVGFAWKYRASNK-DAKYSPNWSHSNKVAVVVVVPILIIIFLAVLTWKTTHALEPSKPL   | 120       |
|          | B.subtilis caa3                                   | TVLSTLIMVVVVAVVSVIFFYVIVRFRRSRVGENTIPKQVEGNKFLEITWTVIPILLLIILVIPVVLYTLELADTSPM  | 120       |
|          | M.tuberculosis                                    | WIGAVIASLAVGVIVWGLIFWSAVFHRKKNT-DTELPRQFGYNMPLELVLTVIPFLIISVLFYFTVVVQEKMLQIAKD  | 155<br>39 |
|          | S.acidocaldarius SoxH                             | MERGRIL LTTIVLAAVVLIVLGVISDGYLALINSGNYL   | 39<br>103 |
|          | R.marinus caa3                                    | FHFWTLVSAIIFVGVVGAMTFFVVRYRRRRRDEVPEPVQEKKVV@LAWIVVPTILVLIVFAWGFRVYIKMYTAPPD  |           |
| A2       | T.thermophilus caa3                               | FPWVYFFSFLIFLVVAGSLAYVTWKFRARPE-DQEEPPQIHGNDRLVVWTLIPLAIVFVLFGLTAKALIQVNRPIPG<br>FNYMMTIATGLFLLVEGVLVYCLIRFRRRKD-DQTDGPPIEGNVPLILWTAIPTVIVFTLAVYSFEVYNNLGGLDPT  | 121       |
| AZ       | Sy.sp<br>A.sp                                     | FNTMMTTAGEFLEVEGVLVYCVVKYRRAG-DHEDGPPVEGNVPL ILWTAIPAIIVIGISVYSFEVYNDIGGFDPH  | 121       |
|          | Aq.aeolicus B1                                    | VTVWLIVAVVIYLIVAIPGIYFIYKYRYKPGEREIGDHDFEGHMGL ALLTIIPLLTVIFLATYSFTIFKKQRTVPDG  | 93        |
|          | N.pharaonis ba3                                   | MNIHTYEKLWLAAAMVLIVGFIATITYGSVGLGIAMVD  | 38        |
|          | T.thermophilus ba3                                | KOVDEHKAHKAILAY KGWLAFSLAMLFVFIALIAYTLATHTAGVIP   |           |
|          | B.staerothermophilus ba3                          | MHIHKY KIWLTFGIGCLVVFLTVVGVSAFAAGQQPPS  |           |
| в        | S.acidocaldarius SoxA                             | MDIKEHAE WWFIVMLVLIFFSWNVYYLSTGKSFSLDY  |           |
|          | Aq.aeolicus B2<br>A.ambivalens                    | MERVTIIGIIFAILVVGWILATGQWAYGNVVGP   | 33        |
|          | A.ambivaiens<br>Ae.pernix 2                       | MARNGLVY YMFIAIIVAVLAGFISSLAYYSYALNVNPAC  | 40        |
|          | -   | I   |           |
|          | P.denitrificans aa3                               | PDL   | 112       |
|          | Bo.taurus   | SLT   | 101       |
| A1       | Ae.pernix 1                                       | DETLYLEKSPPVE   | 124       |
| AI       | E.coli bo3  | AHDERKAE  | 128       |
|          | B.subtilis caa3<br>M.tuberculosis                 | DKKG  | 158       |
|          | S.acidocaldarius SoxH                             | SAO0K0D   | 46        |
|          | R.marinus caa3                                    | AYE   | 106       |
|          | T.thermophilus caa3                               | АМК   | 168       |
| A2       | Sy.sp   | ISRDNAGQQMAHNHMGHMGSMGNMVAMAGDGDVALGIGLDSEEQGVN   | 199       |
|          | A.sp  | AGHQAPITQSSMTMPGSAIAATLNDTPNLNQEKSDEAMQDPATAAVRNADQIPQKQDAPGLGIVAPGIGSSPEKAGKP<br>AMT   | 96        |
|          | L Aq.aeolicus B1<br>N.pharaonis ba3               | DEODTVNPDNLDEHPGFADPGVEQVGE   | 65        |
|          | T.thermophilus ba3                                | AGKLERVDPTTVRQEGPWADPAQAVVQTGP  | 76        |
|          | B.staerothermophilus ba3                          | CLTTIDPEKVDTTPPFDQPGLVKKGE  |           |
| в        | S.acidocaldarius SoxA                             | GLPTYSGLPEQAQKAVQYFDSHPPSPGQYSEVIN  | /4        |
|          | Aq.aeolicus B2                                    |   | 58        |
|          | A.ambivalens<br>Ae.pernix 2                       | LVNHSKIPLLKITTVSATENAAGTT<br>EAIKADQQALEDIMQKAQTTPVVEKAE  |           |
|          | ¢   |   |           |
|          | P.denitrificans aa3                               | VIKAIGHQ <b>MYM</b> SYEYPNDGVAFDALMLEKEALADAGYSE  | 171       |
|          | Bo.taurus   | VIKAIGHQWWWSYEYPNGVAFDALMALEKALA  | 132       |
|          | Ae.pernix 1                                       | DALVVMVIGFQEGEVVTCLN  | 130       |
| A1       | E.coli bo3  | DALVVNVIGFQDGNQEFISVGGEIVIINA<br>KPITIEVVSNDNNKFISVGGEIVIINA<br>DALVVNVRANLAVMEFEYPDYGIITSQ   |           |
|          | B.subtilis caa3                                   |   | 231       |
|          | M.tuberculosis<br>S.acidocaldarius SoxH           | DALVVNVRANLIVNEESIPJGIIISQ<br>  | 71        |
|          | R.marinus caa3                                    |   |           |
|          | T.thermophilus caa3                               |   |           |
| A2       | Sy.sp   |   | ***       |
|          | A.sp  | DELUBRING ON A TRAVER OF A TRAVE OF | 225       |
|          | Aq.aeolicus B1                                    | PELVVNVTGLOUAMIFTYPESGITIG  | 82        |
|          | N.pharaonis ba3                                   | NEYEVHVIAVQVANFPG   | 93        |
|          | T.thermophilus ba3<br>B.staerothermophilus ba3    |   | 81        |
| в        | S.acidocaldarius SoxA                             | GMTJAANU, PATYONKNY PPD   |           |
| D        |   |   | 11        |
|          | Aq.aeolicus B2                                    | MWYRDFNKGAT   | 70        |
|          | A.ambivalens                                      |   | 79        |
|          |   | GVYEVYIVGRQ <mark>B</mark> VMIP   | 79        |

| A1  | P.denitrificans aa3<br>Bo.taurus<br>Ae.pernix 1<br>E.coli bo3<br>B.subtilis caa3<br>M.tuberculosis  | UUU<br>DEYLLATDNPVVVPVGKK-VLVQVTATDVI AWTIPAFAVKQDAVPGRIAQLWFSVDQEGV-YFGQ SIG<br>-LRLLEVDNRVVLPMEMT-IRMLVSSEDVI SWAVPSLGLKTDAIPGRLMQTTLMSSRPGL-YYGQ SIG<br>ELAFPANTP-VYFKVTSNSVMNSFFIPLGSQIYAMAGMQTRLHLIANEPGT-YDGISASYSG<br>ELIVPTDQR-VYFNLKASDVK SFWIPSVGKLDTNTDNENKFFLTPDSKRSKEA-GDMFFGK AG LG<br>TLGTSTEIPVLVLPSGKR-IEFQMASADVI AFWIPELFKRDVMPNPVANNSVNVFQIEEITKTGA-FVGH AG MG<br>  | 241<br>201<br>193<br>212<br>222<br>307<br>132 |
|-----|---|---|---|
| A2  | S.acidocaldarius SoxH<br>R.marinus caa3<br>T.thermophilus caa3<br>Sy.sp<br>A.sp<br>Aq.aeolicus B1   | ELHVPAGQP-VKLRMTSVDVI SFYVPAFRVKQDVLPDRYSALWFEATRPGE-YTVF T Y G<br>ELHVPAGVP-VELEITSKDVI SFWVPGLAGKRDAIFQQTTRISFEPKEPGL-YYGF A L G<br>ELHAPIDRP-VQLNMEAGDVI AFWIPQLRLKQDVIPGRGSTLVFNARHPWA-YPVIAFLG<br>ELHVPIGRE-VQINMTANDVI AFWVPEFRLKQDAIPGRQTEMRFTPKTAGD-YTLIAFLT<br>ELHVPIGRE-VQINMTANDVI SFFVLPARITEDMVFGRTTYLWFQINKPGE-YWF GR Y G   | 189<br>202<br>254<br>286<br>197<br>146        |
| В   | N.pharaonis ba3<br>T.thermophilus ba3<br>B.staerothermophilus ba3<br>S.acidocaldarius SoxA<br>Aq.aeolicus B2<br>A.ambivalens<br>Ae.pernix 2 | DFEVPADSE-VTFHVTSEDVI SFSVVGTNANTMVIPGEVASLTVEMPDVDEPTE-YGIL NY G<br>PIEVPQGAE-IVFKITSPDVI GFHVEGTNINVEVLPGEVSTVRYTFKPPGE-YRII NY G<br>AIEIPKGAK-VTINVTSKDVI GFQIPGTNINMVEPGYVNSLTATFFDEPGE-YTIL NY G<br>LIVVNKSEP-VVLIINSPQUTGFVLRTPDGVINLNNVAGITSYAYFVINQPGH-YTMKDA YAG<br>EIKIPVGSV-VDIFTTSKDVV GVHIHGTNYNVMAIFGTVGYMRIKFEPFGV-YHVV HFG<br>EIYNSTWHVFLYSYNISNDTVKVIQAPWNENKKDYTNWYSGFVVILGSEAQFQ-LQLPFHLSPG<br>STIVLEDPKQ-VTFYVATSDVI GFEIAGTNVNFMVIPGYIAKFTWYPPKNAEGE-YLIL NY G | 142   |
|     |   |   |   |
|     | P.denitrificans aa3<br>Bo.taurus  | IN AY UP IVVKAVSQEKYEAWLAGAKEEFAADASDYLPASPVKLASAE  | 269<br>227                                    |
|     | Ae.pernix 1   | TGBSI WUGKUTUUDKEEFYNAYNSGPDVFSEYVNNVISKYK  | 234   |
| A1  | E.coli bo3  | PCFSCMKFKATATPDRAAFDOWVAKAKOSPNTMSDMAAFEKLAAPSEYNOVEYFSNVKPDLFADVINKFMAHGKSMDM  | 290   |
|     | B.subtilis caa3   | PSTALADFKVKTMSAKEFQGWTKEMKNYKSTAESDLAKQGEELFKEKNCLSCHAVEPNDKRAEAARTAPNLATFGERT  | 370   |
|     | M.tuberculosis  | TY SMMNFEVRVVTPNDFKAYLQQRIDGNTNAEALRAINQPPLAVTTHPFDTRRGELAPQPVG   | 146   |
|     | S.acidocaldarius SoxH   | PG.YTWKGTLIVVS  | 264   |
|     | R.marinus caa3<br>T.thermophilus caa3   | A CEADER FRANKI DEFENDEVEAAKASDADVADERCOOVFOONEAAEHOVARSMPPAVIGPELGLWGNR'   |   |
| A2  | Sy.sp   | AS ARMEER VVVDFREEP DRIVERARASIAT VADBROQUT QUI ATTACTOR AND  | 320   |
| 112 | A.sp  | PYEGAERTOVIVEPEEAFKKWTOEOLAANSDTLNOAVAVNPTDLTPEEFLAPYTKEMGIOPEMLHHIHK   | 355   |
|     | Aq.aeolicus B1  | TO CURRENT ALL DECEMBRIT ACCOUNTS A TRANSPORTED A   | 236   |
|     | N.pharaonis ba3   | PG HDWEALMTVVPEDEFELEDDE  | 168   |
|     | T.thermophilus ba3  | LG QNWFGTIVVKE  | 156   |
|     | B.staerothermophilus ba3<br>S.acidocaldarius SoxA   | AG∰HMUTARIKVVE  | 168   |
| В   | Ag.aeolicus B2  |   | 85  |
|     | A.ambivalens  | TY TT TY TTALYSSKULAKOTATETTS   | 168   |
|     | Ae.pernix 2   | VG QFMKATLIIERSQQALSTSGNSAPEILEYIAGLLSSTTGLALIPPS   | 196   |
|     | -   | 1.  |   |
|     | □ P.denitrificans aa3   | •   |   |
|     | Bo.taurus   |   |   |
|     | Ae.pernix 1   | 315   |   |
| A1  | E.coli bo3<br>B.subtilis caa3   | TQPEGEHSAHEGMEGMDMSHAESAH<br>KVAGVK-EANKENVKAWLKDPDSIKPGNKMTGTYPKLSDSETNALYEYLKGLKAESK <sup>356</sup>   |   |
|     | M.tuberculosis  | KVRGVK-EANKENVRAWDRDI DOTKI GNAMITOTTI REDBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBB  |   |
|     | S.acidocaldarius SoxH   |   |   |
|     | R.marinus caa3  | TTAVAD-ENYLRESILQPGAKVVQGYPNVMPASYASLSEREVAALIEFIKQQQ <sup>316</sup>  | · *   |
|     | T.thermophilus caa3   | SLGAGIVENTPENLKAWIRDPAGMKPGVKMPGFPQLSEEDLDALVRYLEGLKVEGFDFGALPKF- <sup>337</sup>  |   |
| A2  | Sy.sp   |   |   |
|     | A.sp  |   |   |
|     | _ Aq.aeolicus B1<br>□ N.pharaonis ba3   |   |   |
|     | T.thermophilus ba3  |   |   |
|     | B.staerothermophilus ba3  |   |   |
| в   | S.acidocaldarius SoxA   |   |   |
| D   | Aq.aeolicus B2  |   |   |
|     | A.ambivalens  |   |   |
|     | Ae.pernix 2   |   |   |
|     |   |   |   |

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<sub>3</sub>, CAB13363, stearothermophilus ba<sub>3</sub>, BAA31140), *R., Rhodothermuss* (CAC08531), *A., Anabaena* (*Anabaena* sp. strain PCC 7937, CAB10934), *Sy., Synechocystis* (*Synechocystis* sp. strain PCC 6803, S75558), *T., Thermus* (caa<sub>3</sub>, AAA27484, ba<sub>3</sub>, 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<sub>A</sub> 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*<sub>3</sub> 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

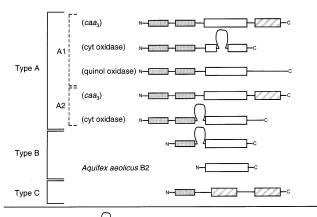
The haem-copper oxidases always contain at least one other subunit, besides subunit I. In types A and B oxidases, this second subunit, named subunit II, is composed of a transmembrane helical domain and a peripheral domain, in the positive (outer or periplasmic) side of the membrane (Fig. 1). The only 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*<sub>3</sub> [53], *A. ambivalens aa*<sub>3</sub> [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 cupredoxinlike fold, built by a 10-stranded  $\beta$ -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, CuA, 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 bo3 oxidase [24,72], amino acid sequence comparisons and structure predictions of the other enzymes (our unpublished data). Several type A oxidases, besides the CuA 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 caa3 oxidases (Fig. 5). The ligands to the Cu<sub>A</sub> 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-



Transmembrane helix  $\Omega$  loop cupredoxin domain  $\square$  haem domain 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*<sub>3</sub> 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<sub>A</sub> (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<sub>3</sub> 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<sub>A</sub>-engineered E. coli bo<sub>3</sub> quinol oxidase [72] and T. thermophilus ba3 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<sub>3</sub> 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*<sub>3</sub> 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 caa3 oxidases [49]). However, a possible relationship between the dihaemic subunit (FixP) and the caa3 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 CuA 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<sub>A</sub> domain has a cupredoxin-like fold.

#### 4. Evolution of terminal oxidases

In the previous sections it was shown that terminal

| C      | Az.caulinodans FixP<br>Br.japonicum FixP<br>Rh.meliloti FixP<br>Rhd.capsulatus FixP<br>P.denitrificans FixP<br>H.pylori FixP<br>R.marinus caa3<br>T.thermophilus caa3<br>B.firmus caa3<br>B.staerothermophilus caa3<br>B.subtilis caa3<br>B.subtilis caa3<br>B.PS3 caa3           |  |
|--------|---|--|
| C<br>A | Az.caulinodans FixP<br>Br.japonicum FixP<br>Rh.meliloti FixP<br>Rhd.capsulatus FixP<br>P.denitrificans FixP<br>H.pylori FixP<br>R.marinus caa3<br>T.thermophilus caa3<br>B.firmus caa3<br>B.staerothermophilus caa3<br>B.staerothermophilus caa3<br>B.subtilis caa3<br>B.PS3 caa3 | LNNPLERWWLWTFYAT I DAFGYVAYPAWPLVSN-YTSGVLGWNSRSAVVEQISDLQKLRAASSAKLANVPLEDIEKNPE <sup>109</sup><br>LNTPLERWWVICFYLT VWAIGY-WIVYPAWPLISS-NTTGLFGYSSRADVAVELANLEKIRGDKMAAGAASLADVEKDPA <sup>105</sup><br>LNNPM RWWVSFYAT I DAIGYAIAYPSWPMLTE-ATKGMLGYSSRAEVSVELAAKAAQAGNLEQIASSSVEEIIANPE <sup>109</sup><br>LNTPLERWWLWTFYAT IDGVAYSIAMPAWPIFSDKATPGLLGSSTRADVEKDIAKFAEMNKAVGEKLVATDLTATAADPE <sup>104</sup><br>YDMPLERWWLWTFYAT UWGVLYLIAYPAIPLVNG-ATQGLLGONYRSDVAAEIQFNEANAPIQAKLVETPLEEIAADPE <sup>105</sup><br>FANNYGVGWLASFMCT VWAFWYFFFGYLNSFGFRVYIKMYTAPPDAYEILVHGYQWYWEFEYPN-GVKTT <sup>127</sup><br>EDQEEPQIHGDRLEVWTLIPLIAYFVLFGLTAKALIQVNRPIPGAMKVEVTGYQFWWDFHYPELGLRNS <sup>140</sup><br>GDDEIKQVHGNTALE TWTVIPIILLUILAVPTIGTFMFADKDPDEVGDNTVYIKVTGHQFWWQFDYENGGFTAG <sup>133</sup><br>GENKI KQVEGNKFLE INTVIPIILLLILAVPTVA-TFFKLADVKPNMDKNR-DKDTVVVNVRANLYWWEFEYPDYGIITS <sup>156</sup><br>EENKI KQVEGSHKLE INTVIPIILLLILAVPTVLTTFKLADVKAMNDKKR-DKNTVVNVRANLYWWEFEYPDYGIITS <sup>156</sup>   |
| C      | Az.caulinodans FixP<br>Br.japonicum FixP<br>Rh.meliloti FixP<br>Rhd.capsulatus FixP<br>P.denitrificans FixP<br>H.pylori FixP<br>T.thermophilus caa3<br>B.firmus caa3<br>B.staerothermophilus caa3<br>B.subtilis caa3<br>B.sys3 caa3   | LISLARAEGKVAF-ADN APELGAGGGGAK FPNLNDDDWLWGGTLAQLQTITHGIRSGDDEGHQGNGLAFG-SI <sup>183</sup><br>LLALARAKGKTVF-GDK APELGSGGAGGK FPNLNDDDWLWGGTLQQTITHGIRSGDDEGHQGNGLAFG-SI <sup>183</sup><br>LLALARAKGKTVF-GDK APELGSGAGGG FPNLNDDDWLWGGTLQQINQTIQFGARSGHAKTHEGQLAFGKDGV <sup>181</sup><br>LQVTYTNAGAAVF-RTW AQCIGAGAGGNT FPSLLDGDWLWGGKPQEIYQTIAHGVRHAPDETRVSDPFFG-DM <sup>181</sup><br>LVTYTRNAGAAIF-RTW AQCIGAGAGGNT FPSLLDGDWLWGGKPQEIYQTIAHGVRHAPDETRVSDPFFG-DM <sup>181</sup><br>LWTYTNAGAAIF-RTW AQCIGAGAGGNT FPSLLDGDWLWGGTLEEIHTTVMHGIRDPKDADTRYSDPFFGIDGL <sup>240</sup><br>LVDMGQGIF-LVHSOCGGGTAEGLHSAQULVRWGKEEGIMDTIHGSKGMDYLAGGPAMELDEK <sup>182</sup><br>NELHVPAGVPVKLBITSKDVIS FFVVPAFRVKQDULPDRYSALWFEATRPGYTYGTEYGGTDSG-LAKVIXH <sup>202</sup><br>QDLVVFVELEITSKDVIS FFVVPALG KIDTVPGTTNISFEPKEPGVFKGCAELCGPSHALK-DFKVRPL <sup>237</sup><br>QDLVVFTNEKVYFNLVASDVK SFWIPAVGGKLDTNTDNKNQFWLVFDQKA-TDKAGGVFYGKCAELCGPSHALK-DFKVRPL <sup>237</sup>  |
| C<br>A | R.denitrificans FixP<br>H.pylori FixP<br>R.marinus caa3<br>T.thermophilus caa3<br>B.firmus caa2   | LSKADI SNVADYVRSLSGAPGDTPAAKKGAE FA-AN ATCHGENGKGNQELGSKNLTDGIWLYGGDKA <sup>252</sup><br>LKGDEIVTVANYVRSLSGLPTRKGYDAAKGEK EV-EN VAC GDGGKGNQEMGAPNLTDGIWLYGGDKA <sup>252</sup><br>LTPELMQQTAAYVVSLJQAPSQPHLVQQ-GKQVFA-DN ASCHGADAKGNREMGAPNLTDGIWLYGGDZ <sup>269</sup><br>LEPAQIDEVVQYVLQISGOPADEVKATA-GQCFA-EN ASCHGADAKGNREMGAPNLTDG   |
| C      | Az.caulinodans FixP<br>Br.japonicum FixP<br>Rh.meliloti FixP<br>Rhd.capsulatus FixP<br>P.denitrificans FixP<br>H.pylori FixP<br>R.marinus caa3<br>T.thermophilus caa3<br>B.staerothermophilus caa3<br>B.staerothermophilus caa3<br>B.subtilis caa3<br>B.PS3 caa3                  | Image: Construction of the second system |

Fig. 6. Amino acid sequence alignment of *caa*<sub>3</sub> oxidase subunit II and dihaemic subunit (FixP) of *cbb*<sub>3</sub> 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* (AAD077216), *R., Rhodothermus* (CAC08531), *T., Thermus* (*caa*<sub>3</sub>, AAA27484), *B., Bacillus* (*firmus caa*<sub>3</sub>, Q04441, *stearothermophilus caa*<sub>3</sub>, BAA11111, *subtilis caa*<sub>3</sub>, CAB13363, PS3, Q03438). Strictly conserved amino acid residues are shaded in black. Cu<sub>A</sub> 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

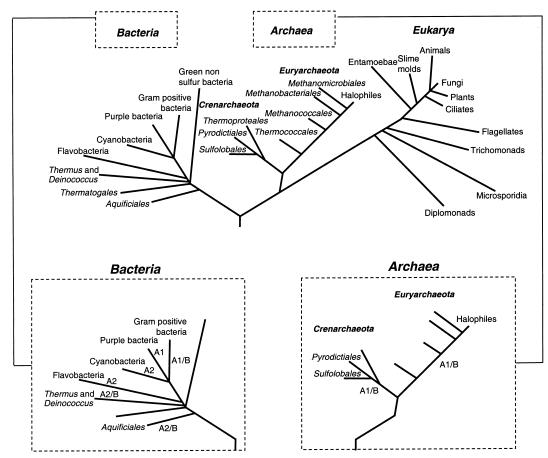


Fig. 7. 16S rRNA phylogenetic tree (adapted from [91]), showing the distribution of A1-, A2- and B-type oxidases, among the different branches of the Bacteria and Archaea domains. In the Bacteria domain (bottom left) the YS motif (type A2) is present in oxidases from bacteria belonging to the deepest 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.

| 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     |

Table 5

Distribution of the three types of haem-copper oxidases among the different domains of life

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 Grampositive 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*<sub>3</sub> 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<sub>2</sub>O 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 haemcopper oxidases evolved from NOR, the cbb3 enzymes being the first oxidases to appear by incorporation of the Cu<sub>B</sub> 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

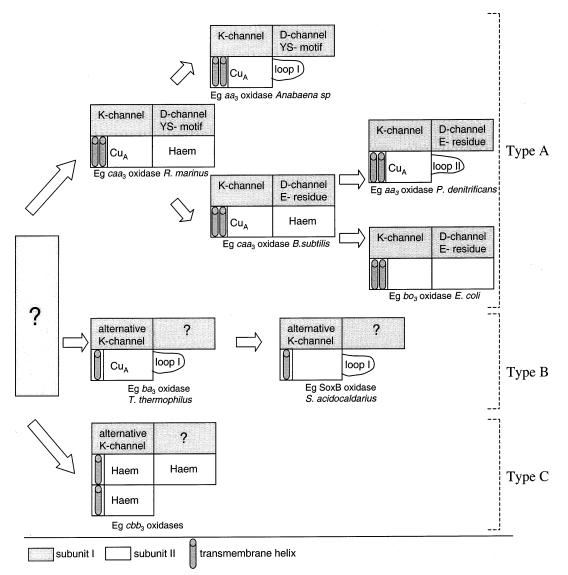


Fig. 8. Parallel evolution of subunits I and II (the minimal functional unit) of terminal oxidases from the three families: types A, B and C oxidases.

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<sub>3</sub> 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<sub>3</sub> oxidase. Two independent occurrences gave rise to the terminal oxidases of cyanobacteria and of the caa3 oxidases from Bacilli. In the case of the cyanobacterial enzymes the event was the loss of the Cterminal 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 bo3 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<sub>A</sub> 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*<sub>3</sub> 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*<sub>3</sub> 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 haemcopper 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 haemcopper 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.

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