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Cytochromes P460 and *c*′-beta; A new family of high-spin cytochromes *c*

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Abstract Cytochromes-P460 of *Nitrosomonas europaea* and *Methylococcus capsulatus* (Bath), and the cytochrome *c*′ of *M. capsulatus*, believed to be involved in binding or transformation of N-oxides, are shown to represent an evolutionarily related new family of monoheme, ~17 kDa, cytochromes *c* found in the genomes of diverse Proteobacteria. All members of this family have a predicted secondary structure predominantly of beta-sheets in contrast to the predominantly alpha-helical cytochromes *c*′ found in photoheterotrophic and denitrifying Proteobacteria.

Keywords: Cytochrome *c*′; Cytochrome P460; Denitrification; N-oxide detoxification; Bacterial enzyme evolution

1. Introduction

Ferrous cytochromes P460 from the autotrophic ammonia-oxidizing bacterium *Nitrosomonas europaea* and the methanotroph *Methylococcus capsulatus* Bath [1] have unique Soret absorbance maxima at 460 nm [2]. Each of the reported 19 kDa subunits in the homodimer has a single pentacoordinate, high spin *c*-heme covalently cross-linked from a porphyrin *meso* carbon to a peptide lysine. The heme binds hydroxylamine, hydrazine, nitrite and cyanide (ferric form) and carbon monoxide (ferrous form) [1–6]. The chromophore of cytochrome P460 resembles the catalytic chromophore, heme P460, of hydroxylamine oxidoreductase from *N. europaea*; a homotrimer of 64 kDa octa-*c*-heme subunits in which the heme P460 is covalently cross-linked to a peptide tyrosine [7,8]. The two proteins have no significant protein sequence similarity [5,9,10]. Cytochrome P460 oxidizes hydroxylamine but does so at a 40-fold lower rate than hydroxylamine oxidoreductase [1,4].

A ~16 kDa, dimeric monoheme cytochrome *c*, co-purifying with *M. capsulatus* cytochrome-P460 [11] was designated a cytochrome *c*′ because its optical and EPR properties and ability to bind NO and CO were similar to the dimeric 4-helical barrel, penta-coordinate cytochromes *c*′ from many photosynthetic and denitrifying bacteria [12–14]. However, the molecular masses (16 kDa versus 12 kDa), redox potential (−205 mV versus −10 to 202 mV) and ligand-binding properties differed somewhat. Cytochrome *c*′ of *M. capsulatus* was reduced in the presence of hydroxylamine and cytochrome-P460 [11].

Protein sequence similarity between cytochromes-P460 of *N. europaea* and *M. capsulatus* and cytochrome *c*′ from *M. capsulatus* suggested common ancestry whereby the acquisition (or loss) of the covalent *c*-heme–lysine cross-link and related ability to oxidize hydroxylamine [15] were the outcome of divergent evolution [16]. Accordingly, cytochrome-P460 was shown to lose its characteristic spectra and enzymatic activity and to acquire cytochrome *c*′-like properties after replacement of the lysine with a non-cross-linking residue [17].

Here, sequence analysis, predicted protein secondary structure, circular dichroism spectroscopy and phylogeny support the designation of a large new "cytochrome P460" family of *c*-cytochromes characterized by a novel beta-sheet structural domain contrasting with the alpha-helical cytochromes *c*′. Two subfamilies have been identified: the heme–lysine cross-linked "cytochromes P460" and the "cytochromes *c*′-beta". A preliminary report of this work has appeared [18].

2. Materials and methods

BLAST and PSI-BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) were performed December, 2006 to identify amino acid sequences similar to those from *M. capsulatus*, *Nitrosococcus oceani*, *Nitrosospira multiformis*, *Nitrosomonas eutropha* and *N. europaea* [19]. After elimination of repetitive sequences, full-length protein sequences were aligned using ClustalX v. 1.83 (Gonnet 250 protein weight matrix with gap opening and gap extension penalties of 35/15 and 0.75/0.35, respectively, for the pairwise/multiple sequence alignments) [20]. Secretion signals and cleavage sites were identified by PSORT (<http://psort.hgc.jp/>) [21].

A total of 58 identified cytochrome *c*′ and P460 protein (or protein domain) sequences were included in alignments and distance neighbor-joining trees were constructed using the BioNJ function in PAUP* v. 4.10b [22]. The trees, together with the placement of the *c*-heme binding motif, were used as guides for manual refinement of the respective ClustalX alignments. Phylogenetic relationships were investigated by character-based tree-searching methods with maximum parsimony or maximum likelihood object functions. A maximum parsimony tree was built from the ClustalX alignment by performing a full heuristic search with the PAUP* program and the following conditions: 50% majority consensus; random order taxon-addition replicates with tree-bisection–reconnection branch-swapping, Mulpars and steepest decent functions in effect. The quality of the branching patterns was assessed by bootstrap re-sampling of the data sets using 1000 replications. We also conducted a maximum likelihood inference by subjecting the alignment to a Bayesian inference of phylogeny using the program MrBayes (v. 3.0b4; <http://morphbank.ebc.uu.se/mrbayes>) [23,24] in which the protein sequence alignment was subjected to

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Metropolis-Coupled Monte Carlo Markov Chain sampling over 1,000,000 generations. The searches were conducted assuming an equal or a gamma distribution of rates across sites and using the WAG empirical amino acid substitution model [25]. A 50% majority rule consensus phylogram was constructed that displayed the mean branch lengths and posterior probability values of the observed clades. Multiplication with 100 made these probability values comparable to the bootstrap proportions calculated for the clade pattern in the maximum parsimony consensus tree.

Secondary structures of mature proteins were predicted by the PSI-PRED server (<http://bioinf.cs.ucl.ac.uk/psipred/>) [26]. The protein sequence logo from relative entropy was generated from the plogo server (<http://www.cbs.dtu.dk/~gorodkin/appl/plogo.html>) [27]. Graphical output was manipulated with Adobe Illustrator software.

The expression and purification of *N. europaea* cyt P460 has been reported elsewhere [28]. CD spectra were collected on a Jasco J-710 spectraphotometer using a 1 cm pathlength quartz cuvette at room temperature. Cytochrome P460 was at 8 μM in 10 mM potassium phosphate buffer at pH 7.0. Spectra were collected at 50 nm/s scan rate, 1 nm bandwidth, and a 2 s response time. Sixteen spectra were averaged and baseline was subtracted. Secondary structure was estimated by fitting a linear combination of α-helix, β-sheet and random coil basis spectra to minimize the root-mean-squared value [29–31].

3. Results

Sequence similarity searches of genomic databases indicated that putative homologues of the cytochromes P460 and *c′* of *N. europaea* and *M. capsulatus* are present in a wide variety but limited number of bacterial species; several being autotrophic ammonia oxidizers or methanotrophs (see Supplementary data for a complete list). Results of BLAST searches using sequences of this family and default parameters did not include members of the four-helix bundle cytochromes *c′*. The more distantly related members of this cytochrome P460 family have ≈20% amino acid sequence identity. Among the six highly conserved and relatively hydrophobic regions (a–f in Fig. 1) were those containing the cross-linked lysine and the heme-binding motif. Few corresponding conserved residues were also present in the alpha-helical cytochromes *c′*.

A predominantly beta-sheet secondary structure was predicted for cytochromes of the P460 family in contrast to the shorter, four-helical structures exemplified by cytochromes *c′* of *Achromobacter xyloseoxidans* and *Rhodopseudomonas palustris* (Fig. 2). Cytochromes of the P460 family are longer than the alpha helical cytochromes *c′*. The validity and applicability of the PSI-PRED algorithm was supported by the excellent correspondence between predicted and X-ray determined secondary structures for the helical cytochromes *c′*, the tetraheme cytochrome *c*554 [32], di heme cytochrome *c* peroxidase [33] and the beta sheet containing cytochrome *f* [34,35]. Fitting of circular dichroism spectroscopy data from the *Nitrosomonas* cyt P460 suggested a 23:44:34 ratio of α-helix:β-sheet:random coil (Fig. 3). Since the analysis is by a technique that does not differentiate well between beta-sheet and random coil, the true amount of beta-sheet will be greater than reported here. PSI-PRED predicted nearly identical secondary structure for 19 other cytochromes of the P460 family (data not shown). Interestingly, four of the five regions of conserved sequence contain five beta sheet and two helical conserved elements of secondary structure; (b) close beta-sheet helix pair, (c–e) three, spaced beta-sheets, (f) the heme-bound helix and a beta sheet

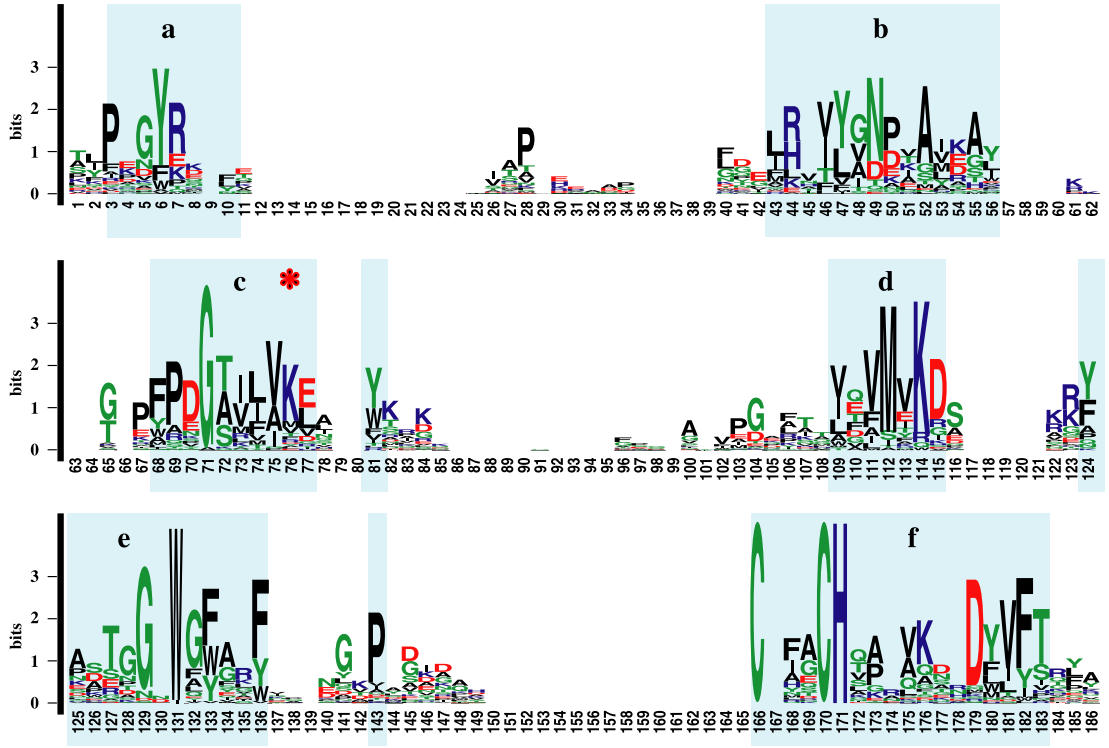

Fig. 1. Sequence logo of the alignment of predicted amino acid sequences in the cytochrome P460 family (The complete alignment and list of strains are in Supplementary data). The height of a residue symbol is proportional to its frequency. The *x*-axis designates residue position in the multiple sequence alignment, and the *y*-axis the information content in bits. Areas of significant sequence conservation were selected as those having a bit score greater than 1.5 and are shaded. The heme cross-linking Lys residue is indicated by an asterisk.

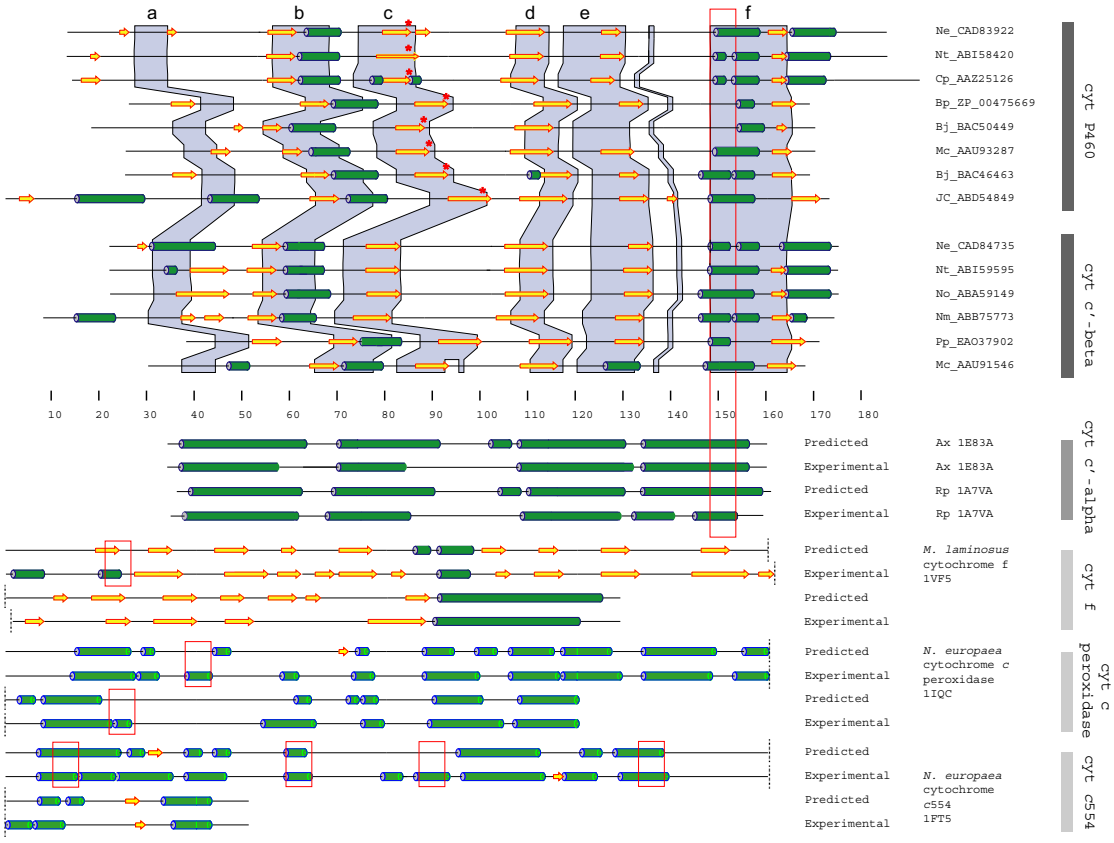

Fig. 2. PSI-PRED-predicted secondary structure of selected members of cytochrome P460 family. Lines indicate random coils, cylinders are alpha-helixes, and arrows are beta-sheets. Sequences are aligned at the heme binding motif, CXXCH, indicated by the red rectangle. Conserved regions, identified in Fig. 1, are shaded and the cross-linking Lys is denoted by asterisks. The numbered scale shows residue number. Predicted and X-ray crystallographically-determined secondary structures are also shown for two four-helix-bundle cytochromes *c*′, a cytochrome *f*, diheme peroxidase, and a tetraheme cytochrome *c*554.

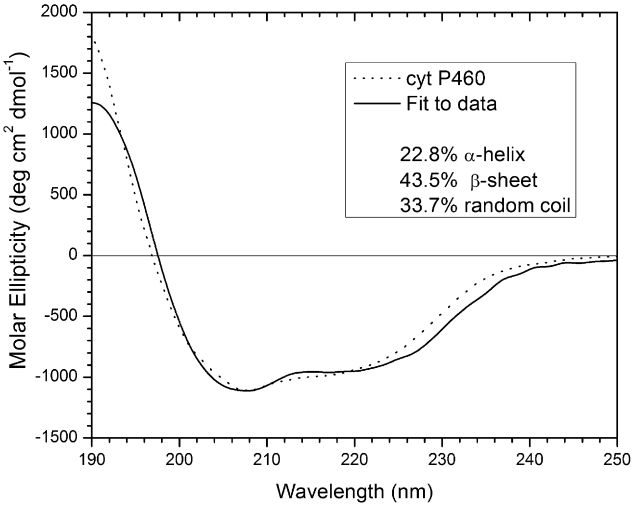

Fig. 3. Circular dichroism spectrum of cytochrome P460 of *Nitrosomonas europaea* and an estimate of the percent secondary structure element.

(Figs. 1 and 2). These five conserved regions might define the protein fold of members of this family.

Refined alignments of cytochromes of the P460 family were used for the inference of phylogeny (Fig. 2 Supplementary data). Sequences containing or lacking, respectively, the homolog of Lys70 (cross-linked to the heme) in *N. europaea*, were considered to be cytochromes-P460 or *c*′-beta. A total of 42 putative cytochromes P460 and 16 cytochromes *c*′-beta (proteins or domains) were distributed in three or two clades, respectively (Fig. 4). Deduced protein sequences from strains in the genera *Kuenenia* (CAJ57118), *Acidobacterium* (ABF42747), *Shewanella* (EAN70732) and *Xanthomonas* (CAJ24025; deleted N-terminus) clustered with and were considered to represent cytochrome P460 protein sequences despite lacking the Lys-70 residue.

Most proteins in the cytochrome P460 family are monoheme and ~17 kDa in mature protein size, with the heme-binding motif near the C-terminus. Multiheme cytochromes *c*, identified in *Solibacter usitatus* Ellin6076 (diheme, EAM57568) and *Kuenenia stuttgartiensis* (triheme, CAJ57118), were divided into C-terminal, mid-protein and N-terminal heme-containing domains (each with a CXXCH motif near the C-terminal end of the domain). In addition, protein sequence ABF51856 from *Sphingopyxis alaskensis* had an N-terminal cytochrome *c*′-beta domain fused to a C-terminal cytochrome c_2 domain. Protein sequences EAQ78521 from *Blastopirullela marina* and EAS60227 from *Flavobacterium johnsoniae* consist of a C-terminal cytochrome P460 domain fused to an N-terminal monoheme domain with high sequence similarity to a yet-uncharacterized small family of putative mono- and di-heme

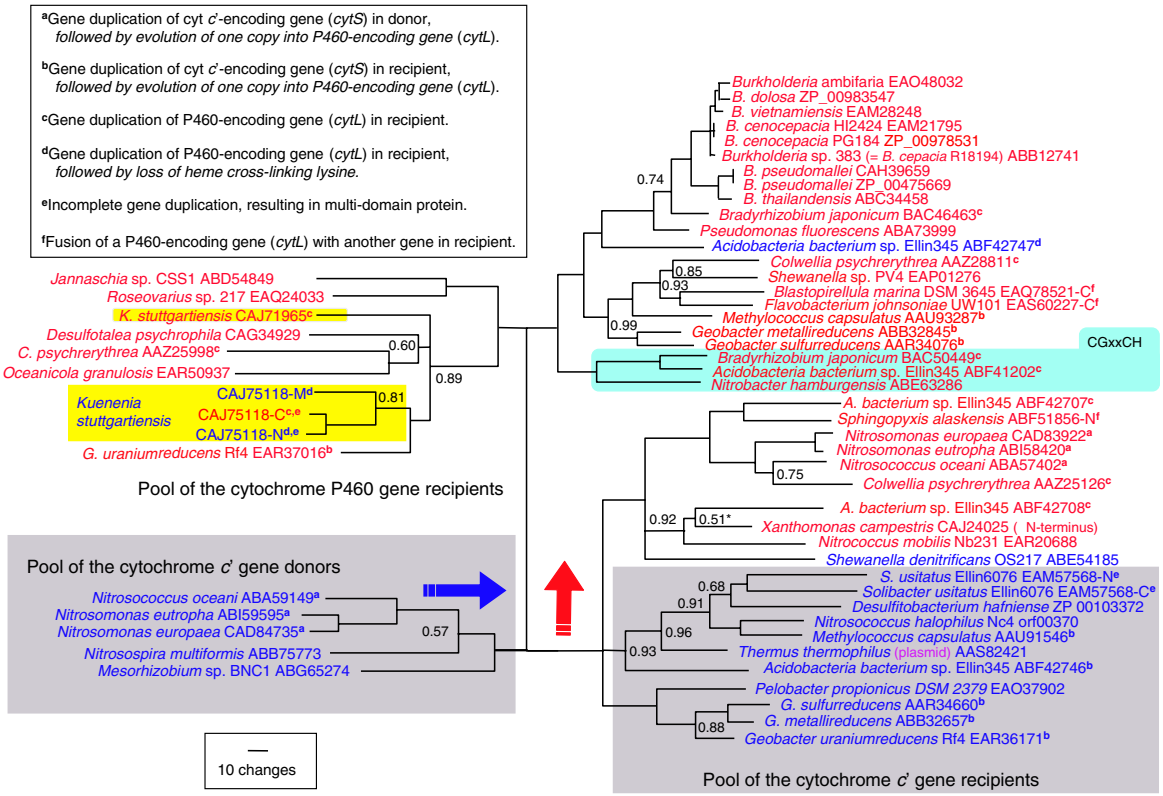

Fig. 4. Unrooted phylogenetic tree constructed from 58 ClustalX-aligned sequences of proteins in the cytochrome P460 family. The hypothesized donor and recipient pools (shaded in dark grey) of the ancestral monoheme cytochrome *c*′ (*cytS*) gene are indicated. Gene transfer and duplication events are indicated by respective a–f as shown in the inset. Posterior probabilities lower than 1.0 are indicated at the branch points. Source organism and protein accession numbers are indicated. Source organisms given in red lettering contain predicted functional cytochrome P460 proteins, whereas blue lettering was used for organisms hosting putative functional cytochromes *c*′ genes as defined by absence of Lys-70. The blue arrow indicates the direction of transfer from the *cytS* donor pool; the red arrow indicates the direction of divergence and evolution of the *cytL* (from *cytS*) genes in recipients before or after lateral transfer processes. Emergence of an unusual heme-binding cassette, CGxxCH, is indicated by teal shading.

cytochromes *c* (ABB24528, ABB23246, AAM72928, AAM-72788, ABB27572, AAF11012, AAS70427 and AAN49274) ("f" in Fig. 4).

4. Discussion

4.1. New cytochrome P460 family

The unusual predominately beta-sheet structure of the *c*-cytochromes typified by the cytochromes P460 and *c*′ of *M. capsulatus* and *N. europaea* contrasts with the four elongate, tightly-packed alpha-helices of the widely distributed cytochromes *c*′ of photoheterotrophic and denitrifying bacteria. We propose that the former be designated the "cytochrome P460 family", comprised of the "cytochromes P460" and the "cytochromes *c*′-beta" defined initially by the presence or absence, respectively, of the heme cross-linked lysine. To remove duplicated acronyms in the literature we rename the genes *cytS*, "Sheet" (formerly *ccp*, Bergmann et al. [16]) and *cytL*, "Lysine" (formerly *cyp*, Bergmann and Hooper [5]). Among known *c*-cytochromes, only cytochrome *f* of photosynthetic bacteria has extensive beta-sheet structure [34], however, it lacks significant sequence similarity to the cytochrome P460 family [35]. We propose to recognize the cytochrome P460 family as a new pfam entry (cytochrome_*C*_3:cytochrome_*c*′-beta and cytochrome P460) within the 72-family cytochromes *c* superfamily and equal in rank to the families pfam00034 (cytochrome_*C*) and pfam012322 (cytochrome_*C*_2:cytochrome_*c*\′ and cytochrome 556) and the remaining 69 families [36]. The prevalence of conserved sequence and hydrophobic residues within the conserved secondary structure elements reinforces the categorization of these proteins as a family. It is also in keeping with a buried hydrophobic core and variable regions closer to solvent, in these soluble proteins.

4.2. Function of members of the cytochrome P460 family

The catalytic or ligand reactivity with nitrogenous oxides by members of the cytochrome P460 family suggests their involvement in the oxidation/reduction (or related electron transfer) or ligation of N-oxides for detoxification or energy generation. This fits with the prevalence of *cytL* and *cytS* genes in denitrifying bacteria, especially in autotrophic ammonia-oxidizing bacteria, which, under oxygen-depleted (e.g., *Nitrosomonas*) or anoxic (e.g., *Kuenenia*) conditions reduce nitrite to NO [37]. By analogy with the alpha-helical cytochromes *c*′ in *Rhodobacter* [14,38] or *Neisseria* in macrophages [39], cytochromes *c*′-beta might sequester or reductively remove nitric oxide to confer resistance to exogenous or endogenously-produced NO. A BLAST search identified *cytL* (but not *cytS* or alpha-helical cytochrome *c*′) genes on the pathogenicity-related smaller lifestyle chromosome II in animal-pathogenic *Burkholderia* species (*Burkholderia cepacia* complex, *Burkholderia pseudomallei* com-

plex) but not in plant-associated or free-living *Burkholderia* strains [40] (URL: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=search&term=txid32008[orgn]>''). This is consistent with a role for cytochrome P460 in resistance to host-generated NO. Cytochrome P460 can oxidize hydroxylamine. In *M. capsulatus*, cytochrome *c*′-beta can act as electron acceptor from hydroxylamine-reduced cytochrome P460 [1]; however, because cytochromes P460 and *c*′-beta are not always found in the same genome, they are not obligatorily redox partners.

4.3. Evolutionary history of the cytochrome P460 family

Cytochromes of the P460 family are present in at least 30 genera of bacteria, most of which are Proteobacteria. Phylogeny of protein sequences of the family (Fig. 4) suggests that cytochrome P460 (*cytL*) genes evolved from ancestral cytochrome *c*′-beta genes (*cytS*) by acquisition of features including the lysine–heme cross-link. This process is analogous to the apparent evolution of a nitrifying hydroxylamine oxidoreductase from an ancestral nitrite-reductase (of the NrfA family) by acquisition of a cross-link from a peptide tyrosine to a ring carbon of the catalytic heme P460 [41].

Our analysis appears to reflect the following evolutionary history: The conserved clustering of the *CytS* and *CytL* protein sequences from the autotrophic ammonia oxidizing bacteria (''a'' in Fig. 4) suggests the origin of a *bona fide cytS* gene in these bacteria followed by a duplication and rapid divergence of one copy into a *cytL* gene. Some species such as *Methylococcus*, *Geobacter* and *Acetobacter* appear to have received ancestral *cytS* from the nitrifiers (''b'' in Fig. 4) prior to duplication and cross-linking (''c'' in Fig. 4). So far, *cytS* and *cytL* genes have been identified as a gene tandem in only one genome (encoding ABF42746 and ABF42747, respectively, in Acidobacteria bacterium sp. Ellin345). Together with the two cases of incomplete gene duplications (*Solibacter*, *Kuenenia*) (''e'' in Fig. 4), these findings are consistent with the scenario of several independent gene duplications followed by genetic drift leading to divergence in sequence and function more than once over a long period of time.

The heme–lysine cross-link of cytochromes P460 may have arisen more than once as indicated by the presence of several separate cytochrome P460 sequence clusters and the distribution of their source organisms. The incongruence of the phylogenetic trees based on the cytochrome P460 family and small subunit ribosomal RNA and the fact that one of the *cytL* genes is plasmid-borne (*Thermus thermophilus*), indicate that the *cytS* and *cytL* genes have arrived independently by horizontal gene transfer in most of the bacterial recipient species and have rarely arisen in a particular bacterial species by a recent gene duplication event (as, for instance, in *N. oceani*).

Uniquely, the *Colwellia* genome lacks a *cytS* gene but contains 3 *cytL* genes, each representing a separate lineage in the tree (Fig. 4) suggesting that the *cytL* gene underwent more than one gene duplication after arrival via horizontal transfer, possibly from a strain related to *N. oceani*. Alternatively, *Colwellia* may have received more than one *cytL* gene in lateral gene transfer events. A somewhat different scenario is presented by the genome of Acidobacteria bacterium sp. Ellin345, which likely received a *cytS* gene by horizontal transfer that then underwent two successive gene duplications. Both genes resulting from the first duplication likely evolved independently into *cytL* genes and duplicated again independently at different times (Fig. 4). One of the second event duplicates (ABF42747) likely underwent a mutation leading to conversion of the critical lysine into an arginine (''d'' in Fig. 4). A similar back-mutation likely led to the loss of the cross-linking lysine in cytochromes P460 from *K. stuttgartiensis* and *S. denitrificans* (''d'' in Fig. 4). Another copy (ABF41202) obtained a codon insertion in the heme-binding motif as did one of the two *B. japonicum cytL* genes (BAC50449), which is also present in the genome of its closest relative, *Nitrobacter hamburgensis*. Other examples of more recent duplications of *cytL* genes include the genes in *Bradyrhizobium* (BAC46463, BAC50449) and *Kuenenia* (CAJ71965, CAJ75118); the latter lost the critical lysine codon and underwent an additional incomplete duplication (Fig. 4).

Multi-domain cytochrome *c* genes found in the genomes of *Solibacter usitatus* Ellin6076 (Fibrobacteres–Acidobacteria group) and in the Planctomycete *Kuenenia* were dividable into two nearly identical domain copies of monoheme cytochromes of the P460 family. We propose that these proteins are the result of incomplete in-frame gene duplications rather than fusions of tandem replicates. The resulting close clustering of the domain sequences in the phylogenetic tree (Fig. 4) suggest that these duplications have occurred fairly recently. The N-terminal extension of the *Kuenenia* cytochrome P460 (CAJ75118C) likely lost the critical lysine codon in the process (CAJ75118M) before undergoing an additional incomplete duplication (CAJ75118N), eventually leading to a triheme cytochrome P460 protein (''e'' in Fig. 4).

4.4. Future work

X-ray crystallography of the structure of cytochrome P460 of *N. europaea*, currently under way [28] , will provide details of the unique protein fold and function of cytochromes of the P460 family. It is hoped that *cyt*S and *cyt*L gene deletion experiments will establish the roles of the cytochrome P460 family proteins *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.01.068.

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