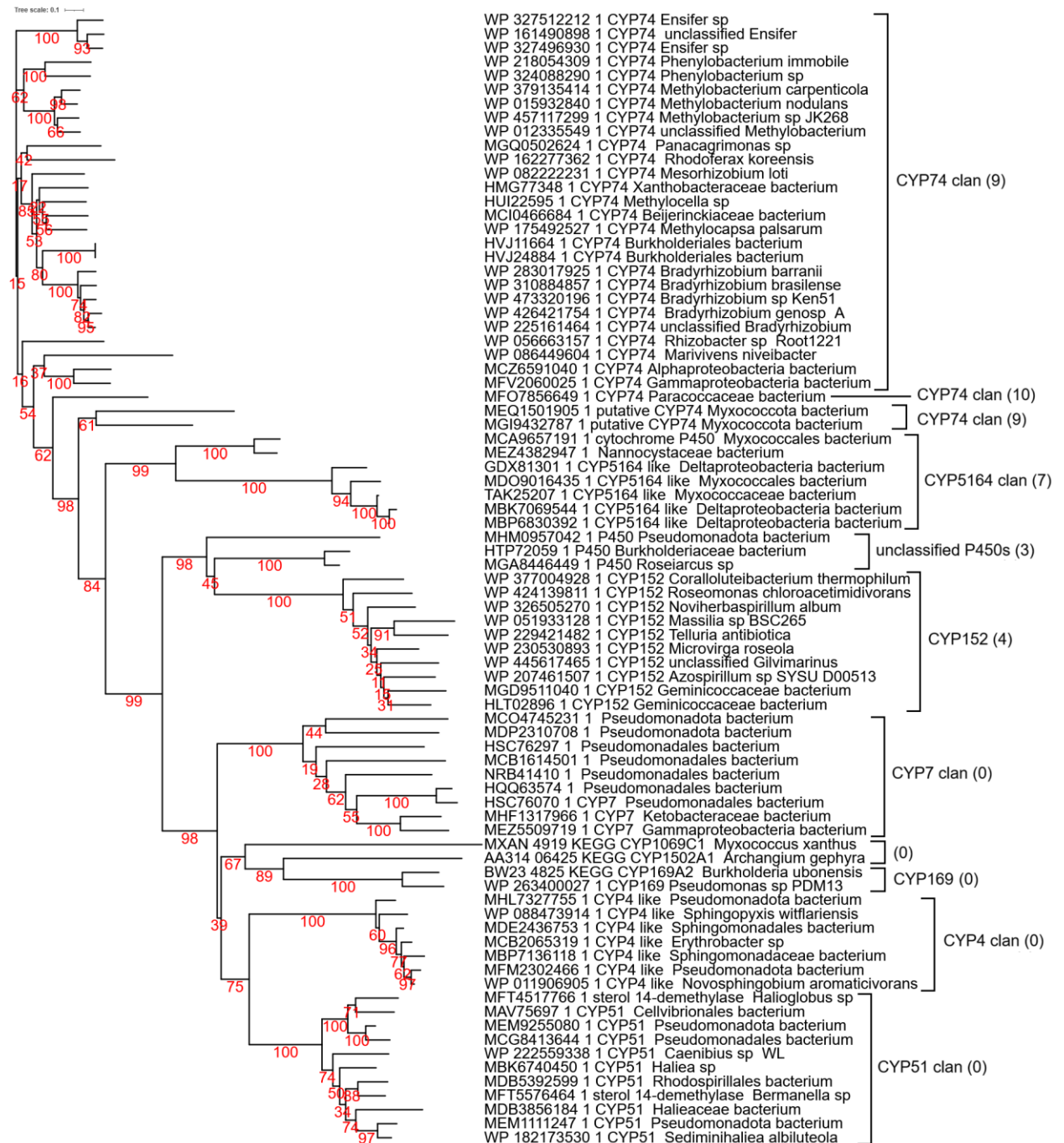


Supplementary Information

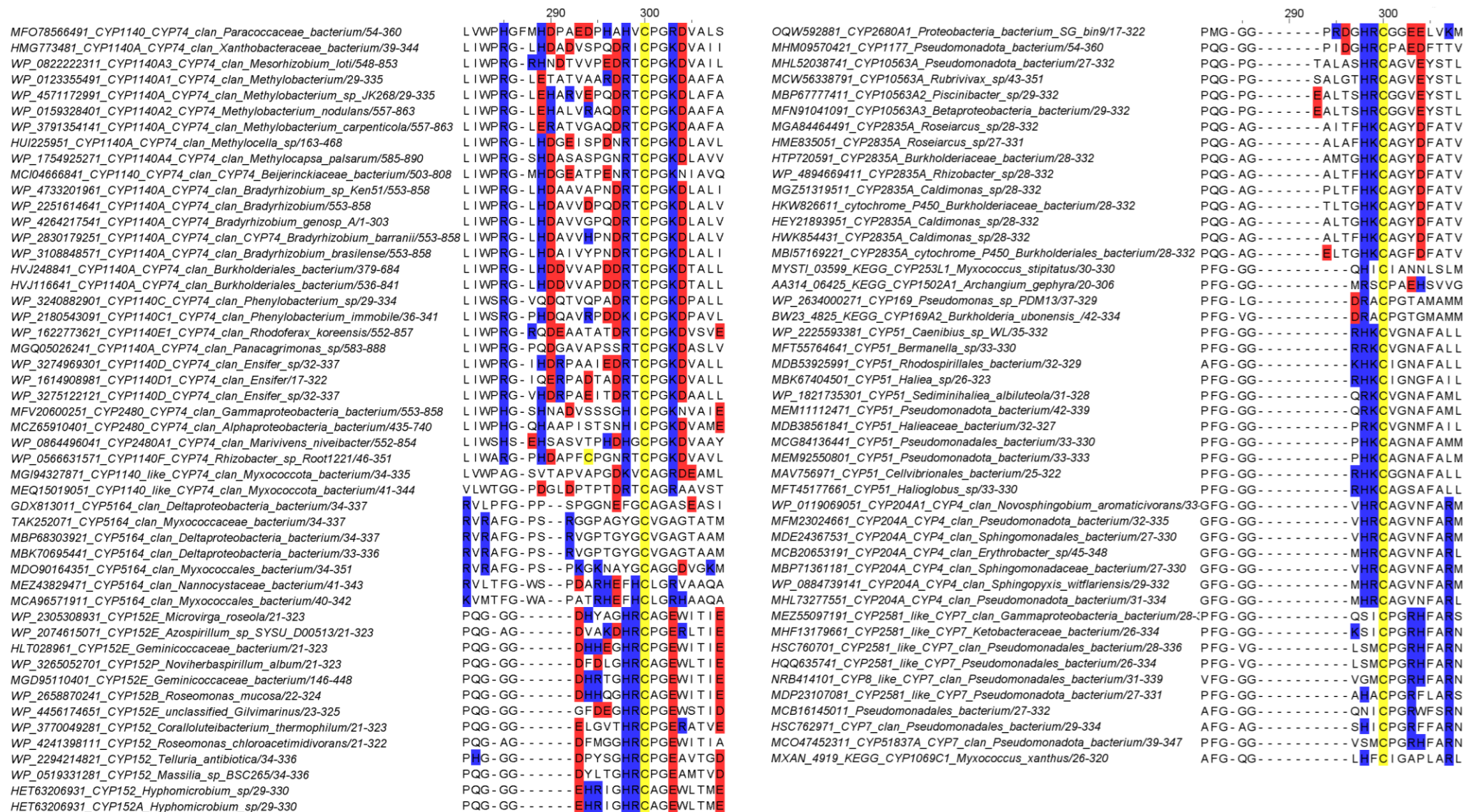
for the article

Proteobacterial CYP74 proteins are extant counterparts of ancestral P450s: phylogenetic and structural insights*

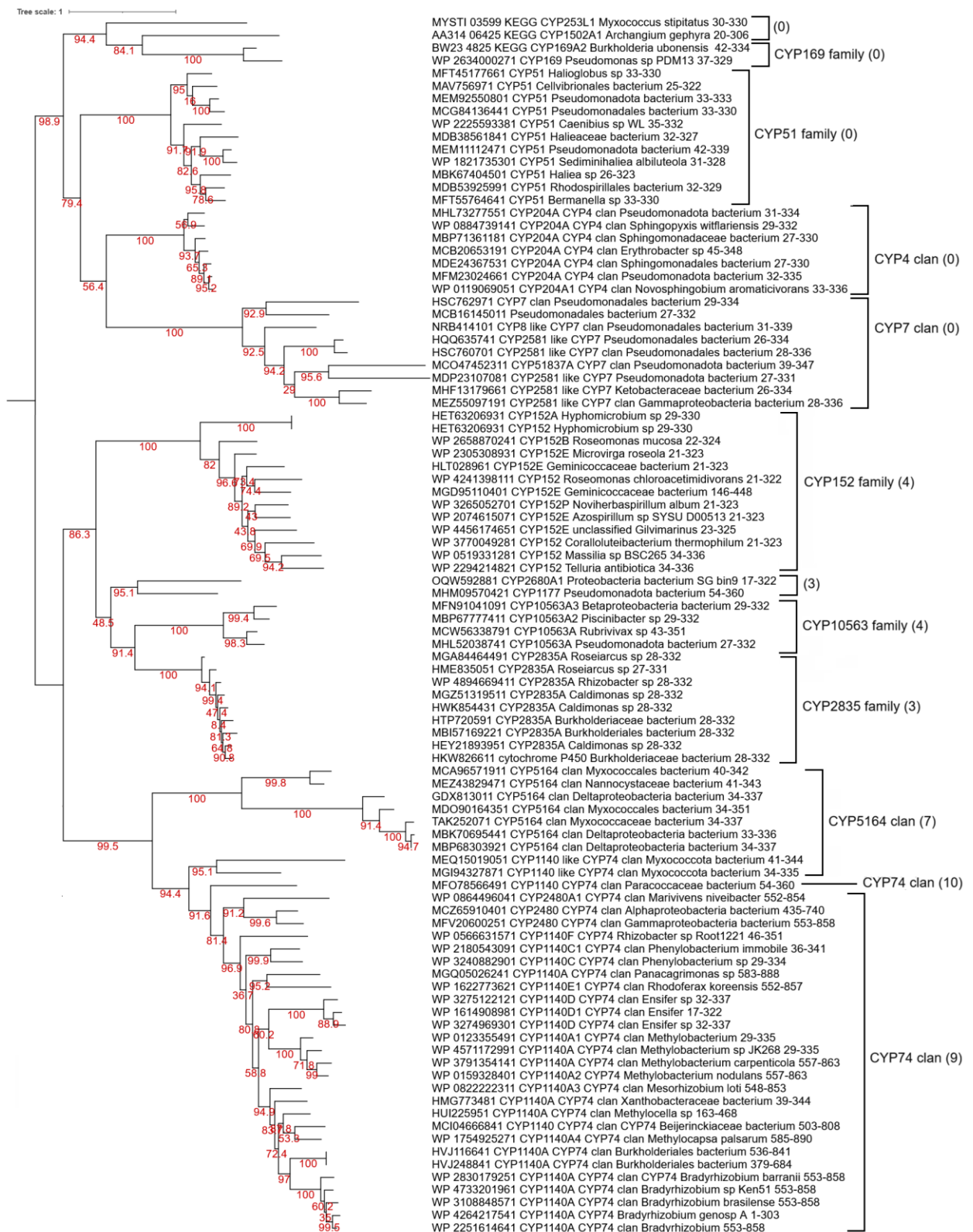
by Alexander N. Grechkin



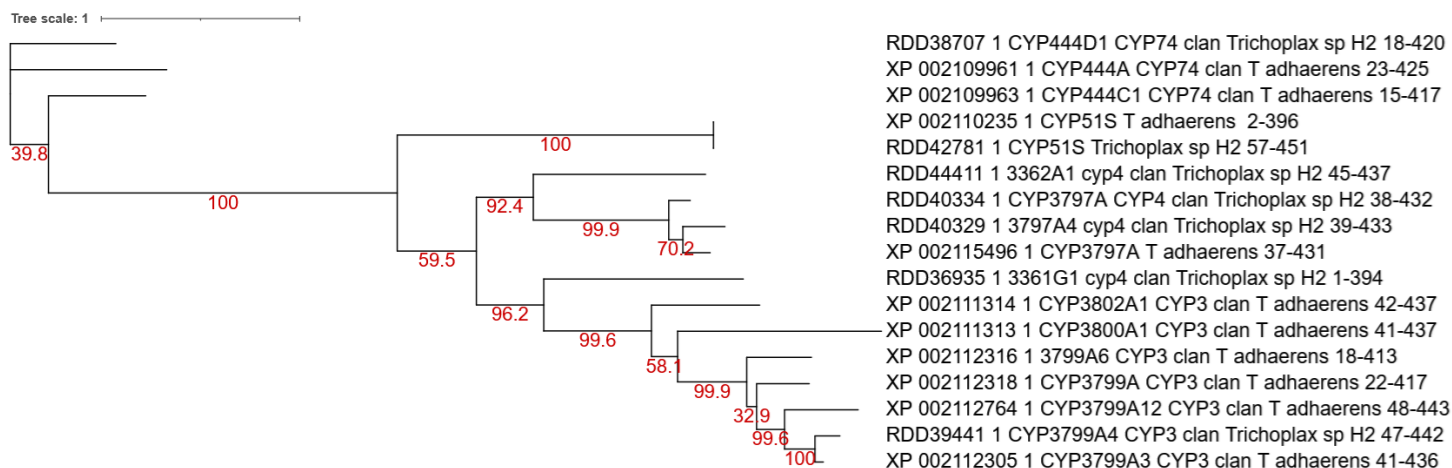
Supplementary Figure S1. Phylogenetic tree of selected proteobacterial P450s. A multiple sequence alignment of 81 proteobacterial P450 proteins was constructed using the MAFFT version 7 web-based platform (Katoh et al., 2019) with the L-INS-i strategy. Gappy regions of the alignment were automatically trimmed by MAFFT software prior the construction of the tree. The phylogenetic tree was constructed using the MAFFT software and the Neighbor-Joining (NJ) method (JTT model; Bootstrap resampling = 100). The tree was visualized with iTOL online software. The red numbers below the branches represent the bootstrap values.



Supplementary Figure S2. Partial multiple alignment of 93 proteobacterial P450s, showing the Cys-pocket region and its insertion with emphasis on charged amino acid residues. Negatively charged residues (D, E) are highlighted in red. Positively charged residues (K, R, H) are highlighted in blue. The cysteine residue having a thiolate bond to haem iron is highlighted in yellow. Left column: the upper part of alignment. Right column: continuation (lower part of alignment). The alignment was built with the MAFFT 7 program and visualized with the Jalview 2.11.5.2 program.



Supplementary Figure S3. Phylogenetic tree of selected proteobacterial P450s. Originally unrooted tree (Fig. 3) was rooted using the midpoint rooting method. A multiple sequence alignment of 97 proteobacterial P450 proteins was constructed using the MAFFT version 7 web-based platform (Kato et al., 2019) with the L-INS-i strategy. Gappy regions of the alignment were trimmed using the Jalview software prior the construction of the tree. The phylogenetic tree was constructed using the W-IQ-Tree program the Maximum Likelihood (ML) method. The tree was visualized with iTOL online software. The red numbers below the branches represent the bootstrap values.



Supplementary Figure S4. Unrooted phylogenetic tree of selected P450s from *Trichoplax* species. Multiple sequence alignments of selected P450s from *Trichoplax* species were generated using the MAFFT v7.526 online program (Kato et al., 2019). Gappy regions of the alignment were manually trimmed with Jalview version 2.11.5.1 (Waterhouse et al., 2009). The resulting gap-free alignments were used as inputs to construct the maximum likelihood (ML) phylogenetic trees with the W-IQ-TREE v2, employing ultrafast bootstrapping procedures. Tree was visualized using the Interactive Tree of Life (iTOL) v7 online program (Letunic and Bork, 2024). The red numbers below the branches represent the bootstrap values.

Supplementary Discussion

The CYP51 clan proteins are often considered the ancestors of the most eukaryotic P450s (Yoshida et al., 2000; Nelson and Nebert, 2018). The CYP51s, certainly, played the ancestral role for a large diversity of eukaryotic P450 monooxygenases. However, the CYP51s were evolved very early in Proteobacteria. Furthermore, they were not the ancestral P450s in Proteobacteria. A substantial diversity of P450s emerged from ancestral proteins in Proteobacteria before eukaryogenesis, as seen from Nzuza et al. (2021), Msomi et al. (2021), and Msweli et al. (2022), as well as from the results of this work. Among the P450s from the Proteobacteria phylum, CYP51s are located at the top of the phylogenetic tree. As noted by McLean et al. (2015), “although CYP51 is likely an early P450, it is not the progenitor in the P450 gene superfamily.” As proposed previously, the hypothetical P450 progenitor was involved in metabolism that was not dependent on molecular oxygen and redox partners (Kahn and Durst, 2000; Werck-Reichhart and Feyereisen, 2000; McLean et al., 2015).

As mentioned in the main text of this article, many proteobacterial CYP74s are fusion proteins. The CYP74 clan domain from the *Methylobacterium nodulans* fusion protein has been cloned and identified as the hydroperoxide lyase (Lee et al., 2008). The full-length DNA has been prepared by PCR amplification and sequenced to confirm the structure (Lee et al., 2008). However, the peroxidase (cyclooxygenase-like) domain has not been cloned, and the recombinant proteins have not been studied. The *M. nodulans* hydroperoxide lyase (Lee et al., 2008) remains the only proteobacterial CYP74 cloned and characterized to date. Nevertheless, the results obtained with the *M. nodulans* enzyme, as well as its fusion structure (including the peroxidase presumably functioning as a dioxygenase), indicate the probable cooperative role of two domains in oxylipin biosynthesis. Thus, most likely, the proteobacterial CYP74s are involved in oxylipin biosynthesis in Proteobacteria.

Supplementary Reference

Nelson DR, Nebert DW (2018) Cytochrome P450 (CYP) gene superfamily. In: eLS. John Wiley & Sons Ltd, Chichester, pp 1-19. <https://doi.org/10.1002/9780470015902.a0005667.pub3>

*The original version of this preprint, dated 20 July 2026, was titled “Phylogenetic and Structural Studies Uncover the Ancestral Role of CYP74 Clan Proteins among Proteobacterial P450s.”

The FASTA dataset of 97 proteobacterial P450s used to generate the multiple alignments and construct the phylogenetic trees

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