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Research review paper

Bacterial lipoxygenases: Biochemical characteristics, molecular structure and potential applications

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ABSTRACT

Lipoxygenases (LOXs) are enzymes that catalyze dioxygenation of polyunsaturated fatty acids into fatty acid hydroperoxides. The formed fatty acid hydroperoxides are of interest as they can readily be transformed to a number of value-added compounds. LOXs are widely distributed in both eukaryotic and prokaryotic organisms, including humans, animals, plants, fungi and bacteria. Compared to eukaryotic enzymes, bacterial enzymes are typically easier to produce at industrial scale in a heterologous host. However, many bacterial LOXs were only identified relatively recently and their structure and biochemical characteristics have not been extensively studied. A better understanding of bacterial LOXs' structure and characteristics will lead to the wider application of these enzymes in industrial processes. This review focuses on recent findings on the biochemical characteristics of bacterial LOXs in relation to their molecular structure. The basis of LOX catalysis as well as emerging determinants explaining the regio- and enantioselectivity of different LOXs are also summarized and critically reviewed. Clustering and phylogenetic analyses of bacterial LOX sequences were performed. Finally, the improvement of bacterial LOXs by mutagenesis approaches and their application in chemical synthesis are discussed.

1. Introduction

Lipoxygenases (LOXs) are non-heme iron (or in some cases manganese) dependent enzymes that catalyze the regioselective dioxygenation of 1*Z*,4*Z*-pentadiene moieties of polyunsaturated fatty acids (PUFAs) to produce hydroperoxy fatty acids. The regioselective dioxygenation of PUFAs by LOXs has a number of interesting applications in the chemical and food industries because it allows the production of different chemicals depending on the position of the hydroperoxide group. The formed hydroperoxy fatty acids can further (1) be chemically or

Abbreviations: LOX, lipoxygenase; PUFA, polyunsaturated fatty acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; OA, oleic acid; DGLA, dihomo-γ-linolenic acid; PLAT, Polycystin-1, Lipoxygenase, Alpha-Toxin; LH, Lipoxygenase homology; PCET, proton-coupled electron transfer; 9-HPODE, 9-hydroperoxy-10*E*,12*Z*-octadecadienoic acid; 10-HPODE, 10(*E*)-hydroperoxy-8-octadecenoic acid; 10-HODE, 10(*E*)-hydroxy-8-octadecenoic acid; 13-HPODE, 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; 9-HPOTrE, 9-hydroperoxy-10*E*,12*Z*,15*Z*-octadecatrienoic acid; 12-HPOTrE, 12-hydroperoxy-9*Z*,13*E*,15*Z*-octadecatrienoic acid; 13-HPOTrE, 13-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid; 9-HPETE, 9-hydroperoxy-5*Z*,7E,11*Z*,14*Z*-eicosatetraenoic acid; 11-HPETE, 11-hydroperoxy-5*Z*,8*Z*,12*E*,14*Z*-eicosatetraenoic acid; 12-HPETE, 12-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; 9-HPEPE, 9-hydroperoxy-5*Z*,7E,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid; 11-HPEPE, 11-hydroperoxy-5*Z*,8*Z*,12*E*,14*Z*,17*Z*-eicosapentaenoic acid; 12-HPEPE, 12-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*,17*Z*-eicosapentaenoic acid; 14-HPEPE, 14-hydroperoxy-5*Z*,8*Z*,11*Z*,15*E*,17*Z*-eicosapentaenoic acid; 15-HPEPE, 15-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-eicosapentaenoic acid; 11-HPDPE, 11-hydroperoxy-7*Z*,9*E*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid; 14-HPDPE, 14-hydroperoxy-7*Z*,10*Z*,12*E*,16*Z*,19*Z*-docosahexaenoic acid; 17-HPDPE, 17-hydroperoxy-7*Z*,10*Z*,13*Z*,15*E*,19*Z*-docosahexaenoic acid; 11-HPDHE, 11-hydroperoxy-4*Z*,7*Z*,9*E*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid; 14-HPDHE, 14-hydroperoxy-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-docosahexaenoic acid; 17-HPDHE, 17-hydroperoxy-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-docosahexaenoic acid; 15-HPETEME, 15-hydroperoxy eicosatetraenoic methyl ester; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; SD, Sloane determinant; BD1, Borngräber 1 determinant; BD2, Borngräber 2 determinant; CS, Coffa site.

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enzymatically converted into hemiacetals that can then dissociate to yield shorter-chain aldehydes, which are of interest as aroma compounds for the food industry (Gigot et al., 2010); (2) be reduced to hydroxy fatty acids, which are of interest for application in cosmetic products and as chemical intermediates (Song et al., 2013).

LOXs are found in many eukaryotic organisms and have been extensively studied in humans (Kutzner et al., 2020; Singh and Rao, 2019; Zheng et al., 2020), animals (Cebrián-Prats et al., 2017; Çolakoğlu et al., 2018; Isobe et al., 2018), plants (Gardner, 1989; Li et al., 2018; Park et al., 2020; Shi et al., 2020; Tayeb et al., 2017), and fungi (Hamberg et al., 1998; Heshof et al., 2014; Speckbacher et al., 2020; Sugio et al., 2018; Wennman et al., 2016). LOXs are known to be involved in the production of oxylipins and various signaling compounds in eukaryotic organisms. Although the existence of these enzymes in prokaryotic organisms (i.e., bacteria) is known, their structure, biochemical characteristics and physiological role have not been broadly investigated.

LOXs from different sources differ in terms of their substrate preference and regioselectivity. Mammalian LOXs are typically active towards arachidonic acid (AA; C20:4 Δ5,8,11,14) and linoleic acid (LA; C18:2 Δ9,12), some are also active towards α-linolenic acid (ALA; C18:3 Δ9,12,15), eicosapentaenoic acid (EPA; C20:5 Δ5,8,11,14,17) and docosahexaenoic acid (DHA; C22:6 Δ4,7,10,13,16,19). Plant LOXs are mostly active towards LA, ALA and/or γ-linolenic acid (GLA; C18:3 Δ6,9,12), although soybean LOX has also been used to produce fatty acid hydroperoxide derivatives from EPA and DHA (Dobson et al., 2013). Similar to plant LOXs, fungal LOXs are mostly active towards LA and ALA (Karrer and Rühl, 2019; Sugio et al., 2018; Wennman et al., 2015; Wennman and Oliw, 2013), except *Magnaporthe salvinii* LOX, which was reported to be mostly active towards AA, EPA and DHA (Sugio et al., 2018). Bacterial LOXs have been reported to be active towards a broad range of polyunsaturated fatty acids, including LA, ALA, GLA, AA, EPA, DPA and DHA (An et al., 2018a; Deschamps et al., 2016; Goloshchapova et al., 2018; Kim et al., 2022; Kim et al., 2021; Oh et al., 2022; Qi et al., 2020). The wider substrate preference of bacterial LOXs can open a broad range of applications for these enzymes. Moreover, bacterial enzymes are typically preferable for industrial-scale chemical synthesis due to the simplicity of producing them in large amounts using a heterologous expression system. Therefore, there has been an increasing interest in bacterial LOXs over the past decade.

A previous article on bacterial LOXs from 2013 reported 38 bacterial genes encoding for LOXs (Hansen et al., 2013). With the huge progress in the genomics and proteomics fields, >4700 bacterial protein sequences from 1100 species were annotated as LOX or as containing a LOX-like conserved domain, i.e. Lipoxygenase, LH2 (Lipoxygenase homology), PLN02337, PLN02305 or PLN02264 domain, have been published in the National Center for Biotechnology Information (NCBI) database. Nevertheless, very few of them have been thoroughly biochemically characterized. In this review, an overview of the current knowledge of the biochemical characteristics and molecular structure of bacterial LOXs will be provided. Moreover, clustering and phylogeny studies of current bacterial LOXs were performed and the improvement of bacterial LOXs by mutagenesis approaches will be discussed. Finally, their potential applications in industrial processes will be described.

2. Occurrence and classification of lipoxygenases

The occurrence of LOXs in many eukaryotic organisms underlines their biological importance. In mammals, hydroperoxy fatty acids produced by LOXs are precursors to various signaling compounds, such as leukotrienes (Haeggström and Funk, 2011), hepoxilins and lipoxins (Joo and Oh, 2012). These compounds are involved in many biological processes (Kuhn et al., 2015), including epidermal differentiation and skin development (Krieg et al., 2013), cell proliferation and carcinogenesis (Dubois, 2003), inflammation (Serhan and Petasis, 2011), blood pressure regulation and atherosclerosis (Chawengsub et al., 2009; Zhu and Ran, 2012), neurodegeneration (Helgadottir et al., 2004) and some metabolic disorders (Ma et al., 2010). In plants, LOXs in conjunction with hydroperoxide lyases and allene oxide synthases are involved in the production of signaling compounds, e.g. methyl jasmonate, and many volatile fatty acid derivatives, e.g. 1-hexanal, *cis*-3-hexenol, and nonanal (Dudareva et al., 2013). Jasmonate is involved in plant defense mechanisms, stress response, growth and development (Wasternack and Hause, 2013). The volatile fatty acid derivatives provide the characteristic 'fresh green' aroma of fruits and vegetables. In fungi, LOXs are involved in the production of oxygenated polyunsaturated fatty acids (oxylipins), which are known as developmental and host–fungal communication signals (Fischer and Keller, 2016; Niu et al., 2020; Tsitsigiannis and Keller, 2007).

Compared to eukaryotic organisms, very little is known about the occurrence and function of LOXs in bacteria. Based on our bioinformatic analysis, up to now only 2.5% of bacterial species in the NCBI database were identified to contain LOX or LOX-like domains and only a few of them have been biochemically characterized. One of the best characterized bacterial LOXs, *Pseudomonas aeruginosa* LOX, was reported to have a membrane dioxygenase activity that induces hemolysis of red blood cells in human hosts (Banthiya et al., 2015). LOX also plays a role in the pathogenesis of *P. aeruginosa.* It promotes the cell persistence in lung tissues (Morello et al., 2019) and the growth of biofilm in association with the host airway epithelium, suggesting a role for the enzyme in the mediation of bacteria-host interactions during colonization (Deschamps et al., 2016). The exact molecular mechanism for this phenomenon has not yet been discovered, but LOX might be involved in intercellular lipid signaling. An in vitro study indicated that *P. aeruginosa* LOX also exhibits lipoxin synthase activity, leading to the production of lipoxin, an anti-inflammatory mediator, that silences the host immune response (Banthiya et al., 2016). In line with these speculations, it has been proposed on the basis of evolutionary and taxonomic analysis that bacterial LOXs are primarily associated with multicellularity and serve as versatile virulence/symbiosis factors which suppress the host immune response (Kurakin et al., 2020). Moreover, it has been speculated that bacterial LOXs are associated with a broad range of hosts, ranging from coral to plants and humans, which suggests that bacterial LOXs may play an important role in host-microbe interactions and provide cross-kingdom host jumps (Kurakin, 2022).

Enzymes from the LOX family are classified as belonging to EC.1.13.11 (dioxygenases), but many of them have a fourth digit based on substrate specificity and/or regio- or enantioselectivity. The regioselectivity is characterized by the position of the carbon atom counted from the carboxyl terminal at which LOXs oxygenate polyunsaturated fatty acids. In some cases, the name of the preferred substrate is also mentioned, e.g. AA 5-LOX, AA 15-LOX, LA 9-LOX, or LA 13-LOX. Depending on the enantioselectivity of the enzyme, the formed hydroperoxy fatty acid can be predominantly in either the *R*- or *S*- configuration. For example, 15-LOX oxygenates AA at carbon-15 from the carboxy terminal, and when the enantioselectivity is known, it can be specified to 15*R*-LOX or 15*S*-LOX. Finally, some LOXs are also named based on their specific characteristics, i.e. fusion-, mini-, and Mn-LOXs. Fusion-LOXs refer to LOXs that are fused to another enzyme so that they have a dual (bifunctional) activity. These enzymes can be found in several corals (Neau et al., 2009) and Cyanobacteria (Schneider et al., 2007). Mini-LOXs refer to low molecular mass LOXs, which can be found in Cyanobacteria (Andreou et al., 2008; Zheng et al., 2008). Mn-LOXs contain Mn^{2+} instead of non-heme iron and are mostly found in fungi (Hörnsten et al., 2002; Su and Oliw, 1998; Wennman et al., 2016). At first, this classification and nomenclature system provided a simple and useful message. However, it is becoming more confusing with the growing diversity of the LOX family, especially when multiple isoforms of the enzyme are present in the same organism (Brash, 1999; Hayward et al., 2017) or when one enzyme is active towards more than one fatty acid substrate and oxygenates each at a different position. Moreover, the recent increase in available genome and protein sequences raised the

number of putative LOX enzymes, while it is difficult to predict the regioselectivity of dioxygenation only from the sequence. This causes confusion in the naming of the enzymes and there is currently no simple and unifying LOX nomenclature system to overcome these difficulties.

3. Biochemical characteristics of bacterial lipoxygenases

3.1. Crystal structure of bacterial LOXs

Generally, eukaryotic LOXs are composed of a single polypeptide chain that folds into two domains, i.e. an N-terminal Polycystin-1, Lipoxygenase, Alpha-Toxin (PLAT) β-barrel domain and a C-terminal catalytic domain (colored in orange and light blue, respectively, in Fig. 1A, B). The N-terminal β-barrel domain has been reported to function as a membrane binding domain to obtain substrate directly from the membrane (May et al., 2000; Tatulian et al., 1998; Walther et al., 2002). This domain is not essential for the catalytic activity, as some N-terminal truncated mammalian LOXs were catalytically active (Walther et al., 2011; Walther et al., 2002). However, the truncated enzyme species displayed impaired membrane binding properties and altered reaction kinetics, suggesting a potential role of the N-terminal β-barrel domain in the regulation of catalytic turnover (Walther et al., 2011). The catalytic domain is mostly α-helical and contains the non-heme iron cofactor in the active site. The non-heme iron is octahedrally coordinated by a water ligand and conserved amino acid residues, which are three histidines, one asparagine or a fourth histidine, and the carboxylate of the C-terminal residue, which is usually an isoleucine.

At the moment, only two crystal structures of bacterial LOXs have been solved. The crystal structure of *P. aeruginosa* LOX presents only one domain (i.e. the C-terminal catalytic domain) (Banthiya et al., 2016) (Fig. 1C). In this enzyme, the N-terminal β-barrel domain found in eukaryotic LOXs, is substituted by double antiparallel α-helices, formed by the insertion of ~100 amino acid residues (colored in pink in Fig. 1C) (Hansen et al., 2013). The partial or total genetic truncation of these N-terminal α-helices makes the enzyme insoluble (Lu et al., 2016; Lu et al., 2014), suggesting that these N-terminal α-helices are required for the solubilization of the enzyme. The truncation studies also indicated that the N-terminal α-helices reduce the substrate binding affinity of the enzyme, and are beneficial to its catalytic activity and thermostability (Lu et al., 2016).

In contrast, *Cyanothece* sp. PCC 8801 LOX has been identified to have two domains and an N-terminal helical extension (colored in pink in Fig. 1D), reminiscent of the α-helical insertion in *P. aeruginosa.* The C-terminal part was identified as the catalytic domain with the conserved

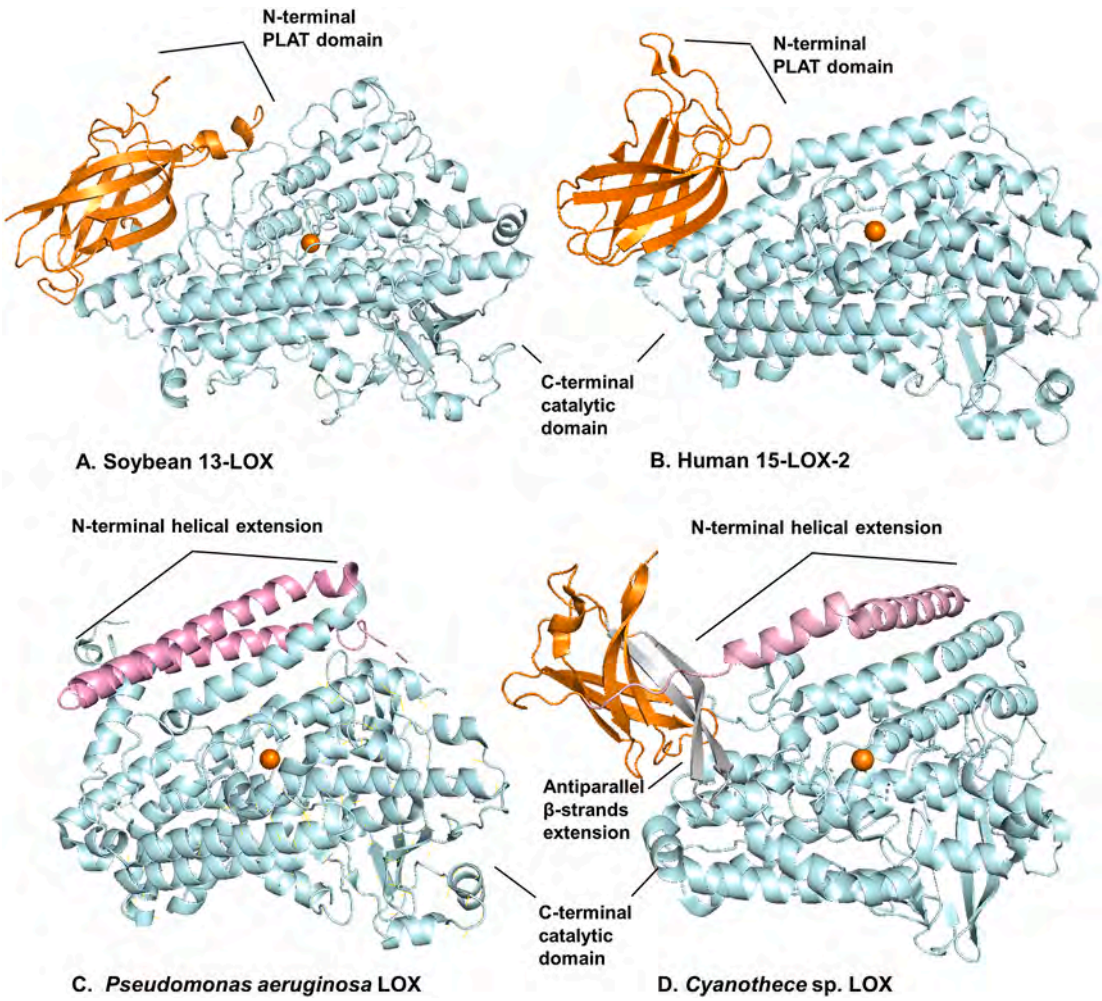

Fig. 1. Crystal structure of two bacterial LOXs compared to eukaryotic LOXs. A) Cartoon representation of soybean 13-LOX. B) Cartoon representation of human 15-LOX-2. C) Cartoon representation of *Pseudomonas aeruginosa* 42A2 LOX. Light pink helices (V114-D206) represent an N-terminal extension, which is not present in eukaryotic LOXs. D) Cartoon representation of *Cyanothece* sp. PCC 8801 LOX. The N-terminal helical extension (M1-A44) is shown in light pink. In each structure, the catalytic non-heme iron ion is shown as an orange sphere, the N-terminal β-barrel PLAT domain is shown in orange, and the C-terminal catalytic domain is shown in light cyan. Figs. A to D were prepared from the PDB files 1IK3, 4NRE, 4G33, and 5EK8 respectively, downloaded from the Protein Data Bank website using PyMOL (Schrödinger LLC, New York, USA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metal ligands His359, His364, His570, Asn574, and Ile668. The N-terminal helical extension is important for membrane binding and its genetic truncation makes the enzyme unable to bind to liposomes (Newie et al., 2016). *Cyanothece* sp. PCC 8801 LOX also includes two antiparallel β-strands, which are extremely extended (colored in light grey in Fig. 1D), resulting in an alteration of the positional arrangement of the β-barrel domain relative to the catalytic domain compared to eukaryotic LOXs (Newie et al., 2016). While in eukaryotic LOX structures, the β-barrel is parallelly oriented to the catalytic domain, in *Cyanothece* sp., it is rotated by >90° (colored in orange). Unlike eukaryotic LOXs, the β-barrel domain of *Cyanothece* sp. LOX does not show a significant role in membrane binding, but is important for the catalytic activity of the enzyme (Newie et al., 2016). An enzyme variant lacking the N-terminal helix, but containing the β-barrel domain, was almost twice as active as the wild-type enzyme, while the truncated protein with both the N-terminal helix and β-barrel domain removed was inactive (Newie et al., 2016).

3.2. Reaction mechanisms

LOXs are non-heme iron (or in some cases manganese) dependent enzymes that catalyze the regioselective dioxygenation of unsaturated fatty acids containing a (1*Z*,4*Z*)-pentadiene structural unit, leading to formation of a conjugated (*Z*,*E*)-hydroperoxydienoic acid. The dioxygenation reaction occurs through four steps (Fig. 2). First, non-heme ferric iron initiates the reaction by abstracting a hydrogen atom stereoselectively at the center of the pentadiene structure through a proton-coupled electron transfer (PCET) mechanism (Lehnert and Solomon, 2003), in which the electron is directly transferred to the ferric iron (Fe^{3+}), and the hydrogen atom removed as a proton to the hydroxide ligand that is coordinated to the iron, yielding ferrous iron (Fe^{2+}), water and a lipid alkyl radical. Second, the lipid radical rearranges into a more stable conjugated diene. In this reaction, the radical electron is dislocated either by a [+2] rearrangement in the direction of the methyl end of the fatty acid or by a [−2] rearrangement in the direction of the carboxyl end. Third, dioxygen is inserted at the side opposite to the removed hydrogen (antarafacially) (Banthiya et al., 2016; Egmond et al., 1972; Hamberg and Samuelsson, 1967; Kalms et al., 2017), producing a lipid peroxyl radical. An exception is formed by the Mn-LOXs of certain fungi, in which the oxygen is inserted suprafacially to the abstracted hydrogen (Hamberg et al., 1998; Wennman et al., 2016). Finally, the lipid peroxyl radical is reduced by ferrous iron and protonated by the iron-bound water molecule to form a lipid hydroperoxide, thus regenerating ferric iron (Fe^{3+}) and the hydroxide ligand for the next cycle of catalysis.

Although most LOXs are only known to convert polyunsaturated fatty acids containing a pentadiene structure, some LOXs have also shown an activity towards monounsaturated fatty acids. *Pseudomonas* sp. 42A2 LOX has been reported to catalyze oxidation of oleic acid (OA) (C18:1 *cis*-Δ9) at C-10 with a simultaneous shift of the double bond to the Δ8 position yielding 10(*E*)-hydroperoxy-8-octadecenoic acid (10-HPODE), which then spontaneously decomposes to the corresponding 10(*E*)-hydroxy-8-octadecenoic acid (10-HODE) (Busquets et al., 2004). Soybean LOX-1 (SBLO-1) has also been reported to catalyze the oxygenation of monounsaturated fatty acids. The monounsaturated fatty acid substrates were initially converted to allylic hydroperoxides before being subsequently converted to the enone products (Clapp et al., 2006). Oleic acid was converted to 11-oxo-9(*Z*)-octadecenoic acid and 12(*Z*)-octadecenoic acid was converted to 13-oxo-11(*E*)-octadecenoic acid plus a minor amount of 11-oxo-12(*Z*)-octadecenoic acid (Clapp et al., 2001). SBLO-1 functionalizes the monounsaturated substrates 4 to 5 orders of magnitude slower compared to LA (Clapp et al., 2006), while *Pseudomonas* sp. 42A2 LOX oxidizes oleic acid at a comparable rate to LA (Busquets et al., 2004). This means that *Pseudomonas* sp. 42A2 LOX will be more applicable for oleic acid hydroxylation.

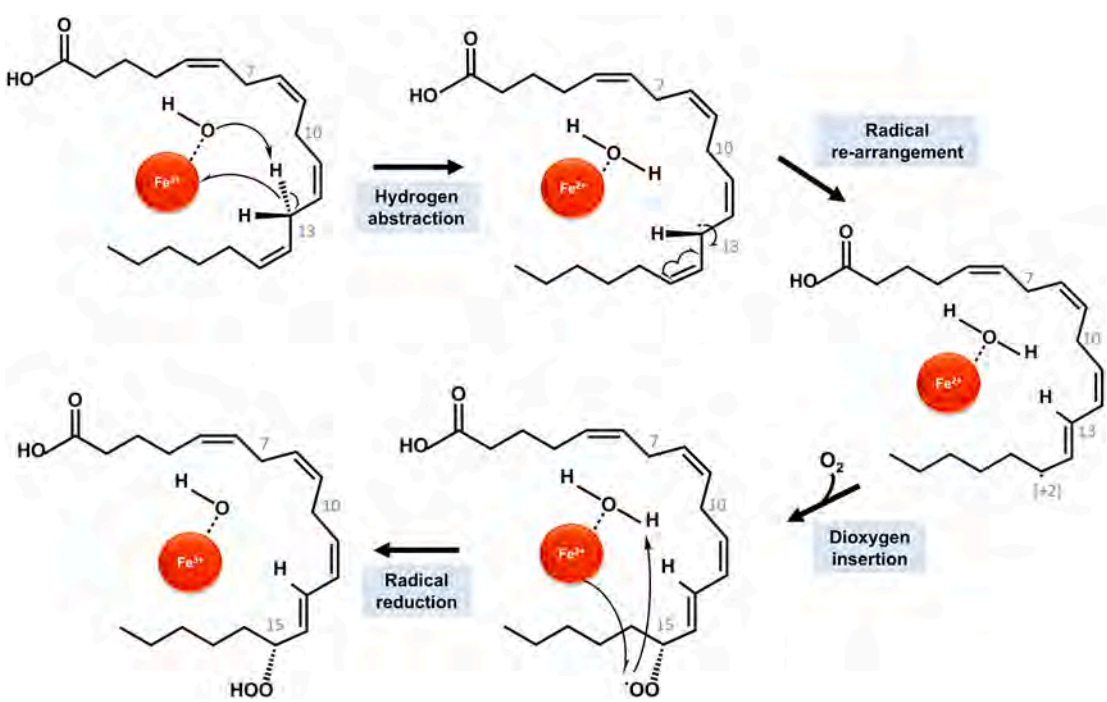

Fig. 2. Reaction mechanism of lipoxygenase (LOX). LOX catalyzes the oxygenation of fatty acids through four reaction steps: (i) The hydrogen atom is stereoselectively abstracted from the center of the reactive pentadiene structure and the resulting electron is transferred to the ferric iron (Fe^{3+}), which is then reduced to the ferrous form (Fe^{2+}); (ii) The radical electron is re-arranged to either the [+2] or the [−2] position from the abstracted hydrogen. This figure illustrates the [+2] re-arrangement; (iii) A dioxygen molecule is introduced at the side opposite to the removed hydrogen generating a fatty acid peroxyl radical; (iv) The fatty acid peroxyl radical is then reduced by an electron from the ferrous iron (Fe^{2+}) and protonated, forming a fatty acid hydroperoxide. Thereby, the iron is re-oxidized to its ferric form (Fe^{3+}) and ready to be used for the next cycle of catalysis.

3.3. Regio- and enantioselectivity

The regioselectivity and enantioselectivity of LOXs are determined by a number of structural features, including (1) the depth of the substrate-binding pocket, (2) the position of the migration channel that shuttles molecular oxygen to the active site (Newcomer and Brash, 2015), (3) the orientation of the substrate in the binding pocket (i.e. carboxy- or methyl-terminal first) (Fig. 3).

The position of the substrate entering the active site cavity is determined by the depth of the binding pocket, which is influenced by the amino acid residues present at the bottom of the pocket (Fig. 3A). When bulky amino acid residues (e.g. phenylalanine, tyrosine, tryptophan) are present at the bottom of the pocket, the substrate will slide into the tunnel in a more superficial way compared to when small amino acid residues (e.g. glycine, alanine, serine) are present. Thus, amino acid residues at the bottom of the binding pocket determine the regioselectivity of oxygenation and their alteration can lead to a change in regioselectivity. To explain regioselectivity, the triad concept was first proposed for mammalian 12/15-LOXs (Vogel et al., 2010) and later on also confirmed for other mammalian 15-LOX orthologs (Heydeck et al., 2022). It suggests that the depth of the binding pocket, and therefore the regioselectivity of the enzyme, depends on three major amino acid determinants: (1) the Sloane determinant (SD) consisting of Ile418 and Met419 (Sloane et al., 1991), (2) the Borngräber 1 determinant (BD1) consisting of Phe353 (Borngräber et al., 1996), and (3) the Borngräber 2 determinant (BD2) corresponding to Ile593 (Borngräber et al., 1999). Alignment of these triad determinants among the LOX superfamily (Fig. 4) showed that in human and mouse LOXs BD1 is mostly occupied by phenylalanine and in some cases by leucine, while these enzymes have very different specificities. Studies of rabbit 15-LOX showed that changing phenylalanine to leucine or alanine without altering the residues at the SD, changes the regioselectivity to 12-LOX (Borngräber et al., 1999; Borngräber et al., 1996). In human and mouse LOX5, BD2 and one of the SD positions are occupied by an alanine, allowing the substrate to slide deeper into the pocket. In mouse ALOX8, one of the SD positions is occupied by serine, a slightly larger residue than alanine, and the BD2 is still occupied by alanine. In human and mouse LOX12, the SD and BD2 positions are occupied by a combination of medium and large residues, i.

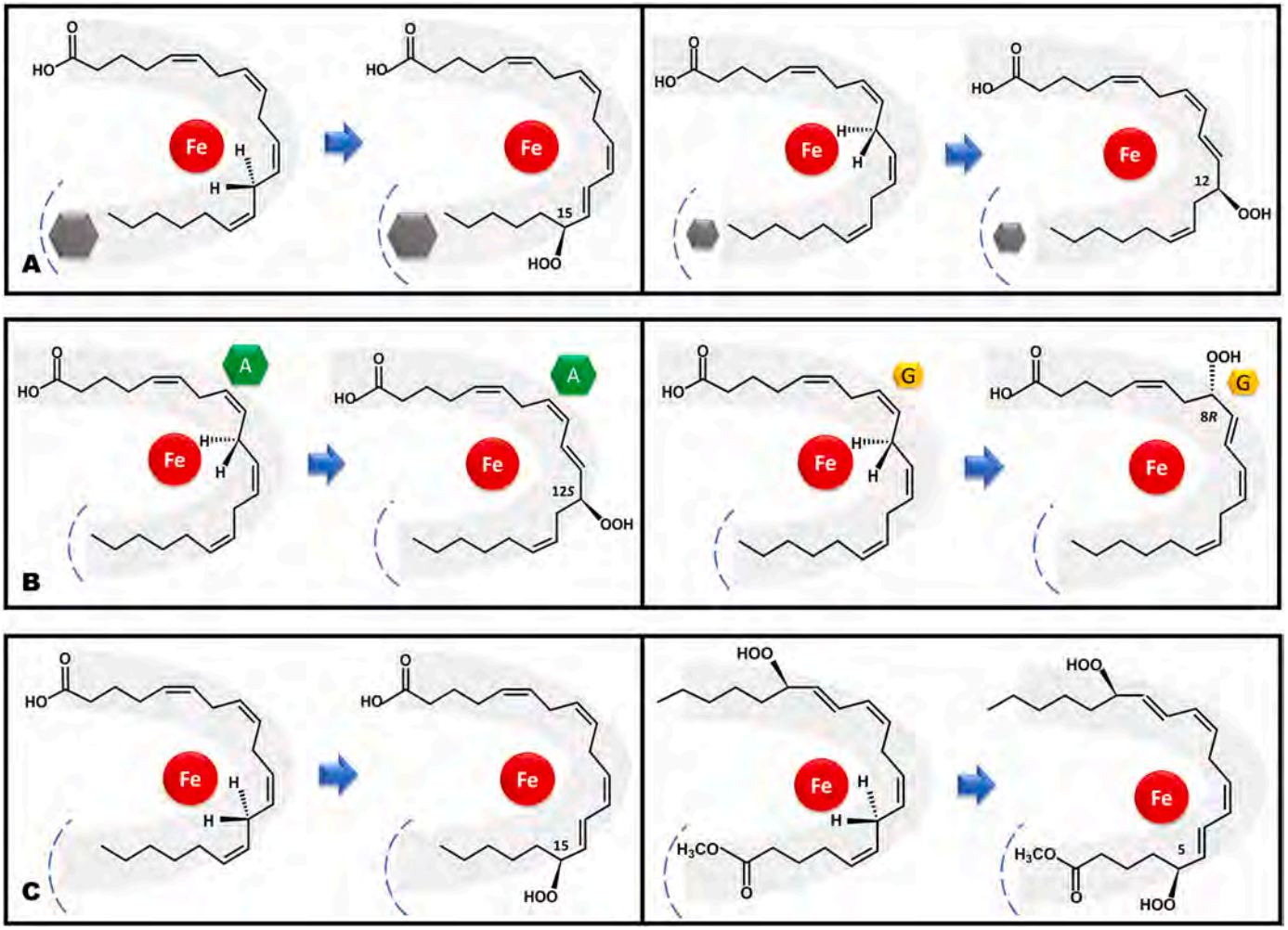

Fig. 3. Mechanisms determining the regio- and enantioselectivity of lipoxygenases (LOXs). The regioselectivity of dioxygenation is determined by 3 main factors. A) The substrate can slide into the binding pocket more or less deeply depending on the amino acid residues at the bottom of the pocket (illustrated by grey hexagons). Bulky residues result in 15*S*-LOX activity (left), while small residues result in 12*S*-LOX activity (right) on an arachidonic acid substrate. B) An amino acid residue in the Coffa site functions as a switch for the dioxygen insertion predominantly at the [+2] or [−2] position from the abstracted hydrogen. The larger alanine residue (green hexagons) accommodates dioxygen insertion at the [+2] position with *S*-specific LOXs, while the small glycine residue (yellow hexagons) accommodates dioxygen insertion predominantly at the [−2] position with R-specific LOXs. C) The orientation of the substrate determines which side of the substrate is exposed to the non-heme iron at the active site. For example, when the methyl-end enters the binding pocket first ("tail first"), it gives a 15-LOX activity on arachidonic acid (left); when the substrate is modified to 15-hydroperoxy eicosatetraenoic methyl ester (15-HPETEME), the methyl ester-end enters the binding pocket ("head-first"), which gives a 5-LOX activity (right) (Schwarz et al., 1998). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

	Enzyme	Accession no.	Reported activity	BD1					CS					SD					BD2				
Mammals	Human LOX5	(P09917)	AA-5S-lipoxygenase	360	D	F	H	V	411	K	A	R	E	425	K	A	N	A	T	604	G	A	V	W
	Human LOX12	(P18054)	AA-12S/15S-lipoxygenase	352	D	F	Q	L	403	R	A	R	T	417	K	A	V	S	T	593	A	I	S	W
	Human LOX12B	(O75342)	AA-12R-lipoxygenase	390	E	F	Y	S	441	I	G	R	A	455	K	G	M	S	L	631	L	V	L	W
	Human LOX15	(P16050)	AA-15S-lipoxygenase	352	D	F	Q	L	403	R	A	R	T	417	Q	I	M	S	T	592	S	I	T	W
	Mouse LOX5	(P48999)	AA-5S-lipoxygenase	360	D	F	H	V	411	K	A	R	E	425	K	A	N	A	T	604	G	A	V	W
	Mouse LOX8	(O35936)	AA-8S-lipoxygenase	366	E	F	Y	I	417	L	A	R	E	431	K	S	T	G	L	607	I	A	L	W
	Mouse LOX12E	(P55249)	AA-12S/13S-lipoxygenase	352	D	F	Q	L	403	L	A	R	N	417	L	V	V	S	T	592	T	V	T	K
	Mouse LX12B	(O70582)	AA-12R-lipoxygenase	390	E	F	Y	S	441	I	G	R	A	455	R	A	M	S	L	631	L	V	L	W
	Mouse LOX15	(P39654)	AA-15S-lipoxygenase	353	D	L	Q	L	404	R	A	R	S	418	K	V	M	S	T	593	N	V	V	W
Plant	*P. homomalla* LOX	(Q27901)	8R-lipoxygenase	400	D	I	T	Y	451	V	G	I	K	465	K	L	F	A	I	643	A	L	S	M
	A. thaliana LOX	(Q06327)	LA-9S-lipoxygenase	511	D	S	G	N	562	L	A	R	Q	576	I	T	V	F	P	771	S	L	I	E
	Soybean LOX1	(P08170)	LA-13S-lipoxygenase	491	D	S	C	Y	542	L	A	R	Q	556	T	T	F	L	P	750	S	V	I	E
Bacteria	*P. aeruginosa* LOXA	(Q9I4G8)	AA-15S-lipoxygenase	369	E	E	N	Y	420	G	A	A	R	434	V	M	F	A	A	608	N	I	Y	H
	P. aeruginosa LOX	(Q8RNT4)	OA-10S/LA-13S-lipoxygenase	369	E	E	N	Y	420	G	A	A	R	434	V	M	F	A	A	608	N	I	Y	H
	B. thailandensis LOX	(Q2SW25)	AA-15S-lipoxygenase	380	E	E	N	Y	431	L	A	A	L	445	T	L	F	A	A	618	A	I	Y	E
	M. fulvus LOX	(A0A511T8A1)	EPA-12S-Lipoxygenase	359	E	G	N	T	410	G	A	R	K	424	D	F	I	A	T	603	A	I	G	R
	Nostoc sp. LOX	(Q8YK97)	LA-9R-lipoxygenase	507	E	F	L	S	558	S	A	V	P	572	I	A	S	M	L	727	S	L	T	W
	Cyanothece sp. LOX	(B7K2Q5)	LA-9R-lipoxygenase	351	A	L	F	D	401	R	G	D	D	416	T	N	G	P	L	573	W	V	N	D

Residue size: XS S M L XL

Fig. 4. Multiple sequence alignment of lipoxygenase superfamily showing Borngräber determinant 1 (BD1), Coffa site (CS), Sloane determinant (SD) and Borngräber determinant 2 (BD2). The reported activity and accession number (UniProt ID) of each enzyme are indicated. The selected residues are colored based on their size, extra-small (XS) with a volume of 60–75 $Å^3$, small (S) with a volume of 76–100 $Å^3$, medium (M) with a volume of 101–140 $Å^3$, large (L) with a volume of 140–180 $Å^3$, and extra-large (XL) with a volume of >181 $Å^3$, displayed as light to dark blue. Multiple sequence alignment was conducted using MUSCLE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

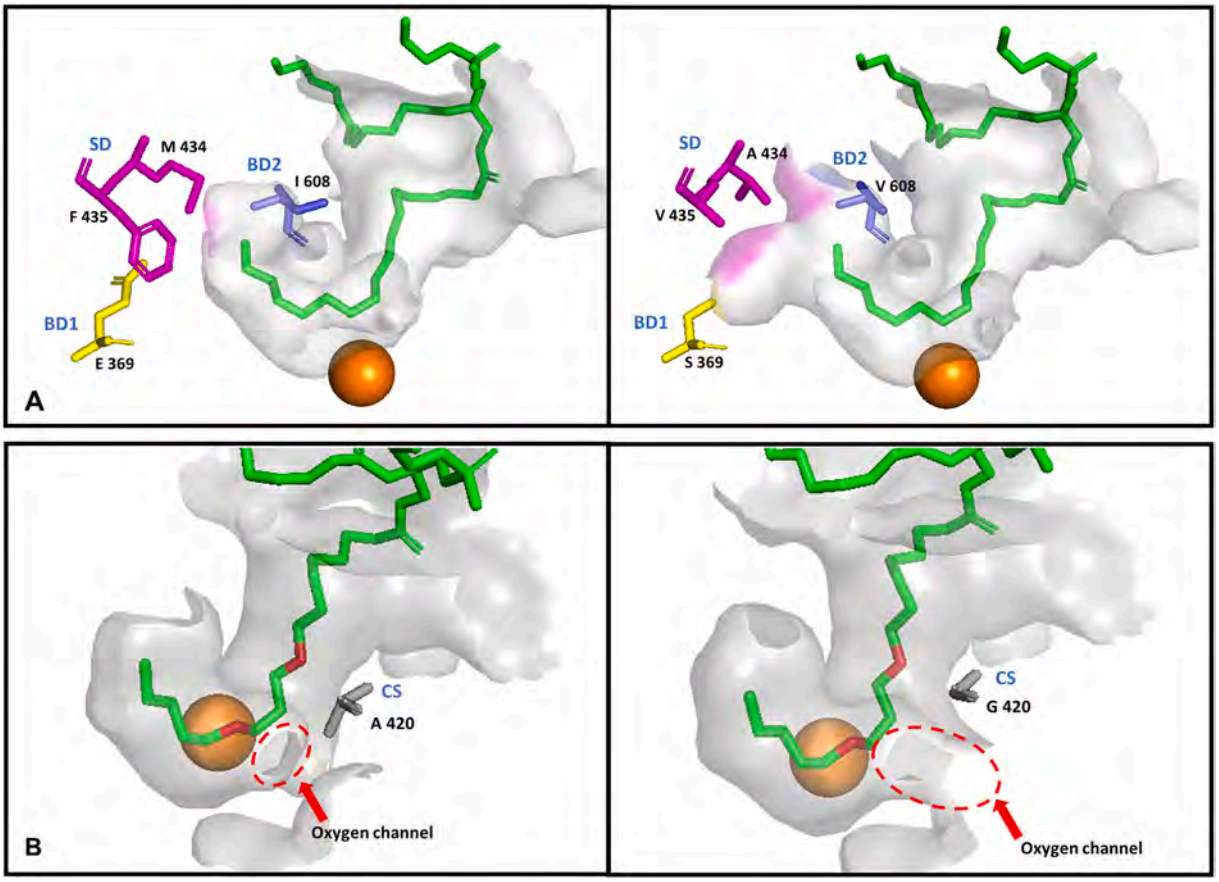

Fig. 5. Binding pocket of *P. aeruginosa* LOX. A) Triad determinants are placed at the bottom of the enzyme binding pocket (left). Simulating mutations exchanging the triad determinants for smaller residues enlarges the enzyme's binding pocket (right). Co-crystalized substrate phosphatidylethanolamine (PE) is shown as green sticks, Borngräber determinant 1 (BD1) as yellow sticks, Sloane determinant (SD) as magenta sticks, and Borngräber determinant 2 (BD2) as blue sticks. The binding pocket is shown as grey shading. B) Alanine (grey sticks) present at the Coffa site (CS) covers part of the oxygen channel area (left), providing a narrow oxygen channel (shown as red dashes). Simulation of the mutation Ala420Gly provides a more spacious oxygen channel (right), allowing the insertion of oxygen in both the [+2] position and the [−2] position from the abstracted hydrogen. Possible oxygen insertion positions in the hydrocarbon chain are shown in red. The figure was prepared from PDB file 5IR5, downloaded from the Protein Data Bank website using PyMOL (Schrödinger LLC, New York, USA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

e. valine and isoleucine. In human and mouse LOX15, the SD and BD2 positions are occupied by large residues, i.e. isoleucine and methionine. It seems there is a sliding scale of binding pocket volume from 5- to 8- to 12- to 15-LOXs. Soybean 13*S*-LOX has a phenylalanine in its SD while *Arabidopsis thaliana* 9S-LOX has a valine. This confirms the role of SD in determining the enzyme regioselectivity.

The triad concept can also clearly be observed in the case of bacterial LOXs. Bacterial LOXs with bulkier residues in their specificity determinants (e.g. *P. aeruginosa* and *B. thailandensis*) add the hydroperoxide group on the edge of the double bond system closer to the methyl end, i.e. AA-15*S*- and LA-13*S*-LOXs (Fig. 4). On the other hand, bacterial LOXs with one or more small residues in their specificity determinants (e.g. *Nostoc* sp., *Cyanothece* sp. PCC 8801, and *M. fulvus*) introduce the hydroperoxide group further away from the methyl group, i.e. LA-9*R*- and EPA-12*S*-LOXs, suggesting that the substrate cannot slide as deeply into the binding pocket (Fig. 4). The triad determinants in the crystal structures of *P. aeruginosa* LOX clearly show that these determinants are present at the bottom of the binding pocket, providing further support for their role in determining the regioselectivity of the enzymes (Fig. 5A).

The regio- and enantioselectivity of LOXs is also influenced by the position of the migration channel that shuttles molecular oxygen to a specific region of the active site. An oxygen migration channel has been reported before for soybean LOX-1 (Knapp et al., 2001; Knapp and Klinman, 2003) and later for rabbit 12/15-LOX (Saam et al., 2007). Single site mutation of Leu496 to Trp in the soybean LOX-1 oxygen channel changes the direction of the oxygen access tunnel towards an alternate position of the substrate, altering the regio- and enantioselectivity of the reaction (Collazo and Klinman, 2016). An alternative mechanism determining the regio- and enantioselectivity of LOXs by directing the oxygen molecule through a glycine/alanine switch in the Coffa site, has been proposed (Coffa and Brash, 2004). When an alanine residue is present in the Coffa site, it covers the migration channel at the [−2] position (from the abstracted hydrogen) and promotes oxygenation at the [+2] position of the reactive pentadiene resulting in formation of the *S*-enantiomer product (Fig. 3B). On the other hand, when a glycine residue is present in the Coffa site, the migration channel becomes more spacious (Fig. 5B) (Kalms et al., 2017), thereby promoting oxygenation both at the [−2] position, resulting in the *R*-enantiomer product, and at the [+2] position, resulting in the *S*-enantiomer product.

The role of the Coffa determinant on the enantioselectivity can be observed in human, mouse, plant and bacterial LOXs (Fig. 4). The importance of the Coffa site for the enantioselectivity of bacterial LOXs was addressed by site-directed mutagenesis (Table 1). In most cases of bacterial LOXs, the changes in enantioselectivity related to changes in the residue at the Coffa site are strictly connected with changes in the regioselectivity. This is in accordance with the previous report on mouse LOX, which changes from an 8*R*- to a 12*S*-lipoxygenase by mutation of the residue at the Coffa site (Coffa et al., 2005; Coffa and Brash, 2004). Thus, the enantiomer shift from *R* to *S* is a side result of the altered regioselectivity, since the oxygen still comes from the same face of the substrate (Coffa et al., 2005). Although the glycine/alanine switch can help determine the enantioselectivity of most LOXs, there are some exceptions, i.e. Zebrafish LOX-1 (Jansen et al., 2011), *Nostoc* sp. mini-LOX (Andreou et al., 2008), and *M. fulvus* MF-LOX1 (Goloshchapova et al., 2018). In *Nostoc* sp. mini-LOX, the oxygen is introduced in the *R*-configuration, while having an alanine at the Coffa site (Fig. 4). Changing the alanine residue in the Coffa site to glycine did not change the enantioselectivity of the enzyme, while replacing it by a bulkier residue, i.e. valine or isoleucine, changed the selectivity of the enzyme from 9*R*-lipoxygenation to almost exclusively 13*S*-lipoxygenation (Andreou et al., 2008). It seems that in this enzyme, alanine is not sufficiently bulky to achieve the effect of blocking the oxygen channel. A similar switch in selectivity such as described for *Nostoc* sp. mini-LOX was also reported for *Anabaena* sp. PCC 7120 LOX, however changing Ala215 to Phe did not change selectivity of the enzyme (Zheng et al., 2008). In *M. fulvus* MF-LOX1, the point mutation Ala410Gly did not affect the regio- and enantioselectivity of the enzyme (Goloshchapova et al., 2018). However, the introduction of bulkier residues than alanine at this site was not explored.

Table 1
Mutagenesis studies at Coffa site of bacterial LOXs.

Source of enzyme	Reported activity	Mutation at Coffa site	Activity of the mutant	Reference
Cyanothece sp.	LA 9*R*-LOX*, LA 13*S*-LOX	Gly401Ala	LA 13*S*-LOX8*, LA 9*R*-LOX	(Newie et al., 2016)
Pseudomonas aeruginosa	LA 13*S*-LOX	Ala402Gly	LA 9*R*-LOX, LA 13*S*-LOX	(Kalms et al., 2017)
	AA 15*S*-LOX		AA 11*R*-LOX*, AA 15*S*-LOX	
Nostoc sp.	LA 9*R*-LOX*, LA 13*S*-LOX	Ala162Gly	LA 9*R*-LOX*, LA 13*S*-LOX	(Andreou et al., 2008)
		Ala162Val	LA 13*S*-LOX*, LA 9*R*-LOX,	
		Ala162Ile	LA 13*S*-LOX*, LA 9*R*-LOX,	
Anabaena sp. PCC 7120	LA 9*R*-LOX*, LA 13*S*-LOX	Ala215Gly	LA 9*R*-LOX*, LA 13*S*-LOX	(Zheng et al., 2008)
		Ala215Val	LA 13*S*-LOX*, LA 9*R*-LOX,	
		Ala215Phe	LA 9*R*-LOX*, LA 13*S*-LOX	
Myxococcus fulvus	AA 12*S*-LOX*, AA 15-LOX	Ala410Gly	AA 12*S*-LOX*, AA 15-LOX	(Goloshchapova et al., 2018)
Oscillatoria nigro-viridis PCC 7112	LA 13*S*-LOX	Ala296Gly	LA 9*R*-LOX	(Yi et al., 2020)

* predominant activity.

The third factor proposed to influence the specificity of hydrogen abstraction and dioxygen insertion is the orientation of the substrate. Conceptually, the fatty acid substrate can slide into the binding pocket either with its methyl terminal (i.e. tail-first) or with its carboxylate group (i.e. head-first) first. This concept was previously used to explain the regioselectivity of the double dioxygenation reactions of soybean LOX-1 on AA, for which the initial product, 15-HPETE, is further oxygenated at a much slower rate to the specific double oxygenation products, 5S,15*S*-di-HPETE and 8*S*,15*S*-di-HPETE (Van Os et al., 1981). Double oxygenating LOXs have also been reported from bacteria, i.e. *Endozoicomonas numazuensis* LOX, which catalyzes 5*S*,12*S*-dioxygenation on AA and EPA as well as 7*S*,14*S*-dioxygenation on DPA and DHA (Kim et al., 2021) and *Archangium violaceum* LOX, which catalyzes 5*S*,15*S*-dioxygenation on AA and EPA as well as 7*S*,17*S*-dioxygenation on DPA and DHA (Lee et al., 2022). These studies demonstrate that the same enzyme is able to catalyze different regioselective dioxygenations, providing evidence that tail-first and head-first binding can occur in the same active site. Further evidence comes from the structures of human 5-LOX and 15-LOX-2. These enzymes have cavities of equal depth,

suggesting that the substrate entry in 15-LOX-2 is tail-first and in 5-LOX is head-first (Newcomer and Brash, 2015). Moreover, the product of oxygenation of LA by soybean LOX-1 switches from 13*S*-HPODE (tail-first binding product) to 9*S*-HPODE (head-first binding product) at lower pH, corresponding to the pH-dependent suppression of carboxylate group ionization. This suggests that the substrate can enter the binding pocket in the head-first orientation only when it is in carboxylic acid form (Gardner, 1989).

The tail-first substrate binding also can be inferred from mutagenesis studies where a change in specificity from 15- to 12-LOX was observed when the depth of the binding pocket was changed, suggesting that the tail is sliding deeper after mutation (Sloane et al., 1991; Vogel et al., 2010). The head-first orientation of the substrate can also be triggered by some conditions, i.e. the use of modified fatty acids as the substrate (Fig. 3C). For example, rabbit and soybean 15-LOX induced the introduction of dioxygen on 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) predominantly at C_5 because C_{15} was already oxygenated (Schwarz et al., 1998). Moreover, modification of the carboxylate of the fatty acid by methyl esterification induces a head-first orientation. This was observed when 15-HETE and 15-HETE methyl ester were used as substrates of soybean 15-LOX, with the latter substrate displaying a higher oxygenation rate at C_5 (Schwarz et al., 1998). Further modification at both ends of the fatty acid by introducing a bulky (-$C(CH_3)_3$) or polar (-OH) residue at the methyl terminus and methylation of the carboxylate (Walther et al., 2001) also increases the chance for a head-first orientation. However, in these cases, the energetic barrier associated with burying the head of the fatty acid in the hydrophobic environment of the substrate-binding pocket is reflected by a strong reduction of binding affinity (i.e. increase in K_M value). The alignment of the substrate in a specific orientation to the active site therefore becomes one of the key factors controlling the specificity of the oxygenation reaction.

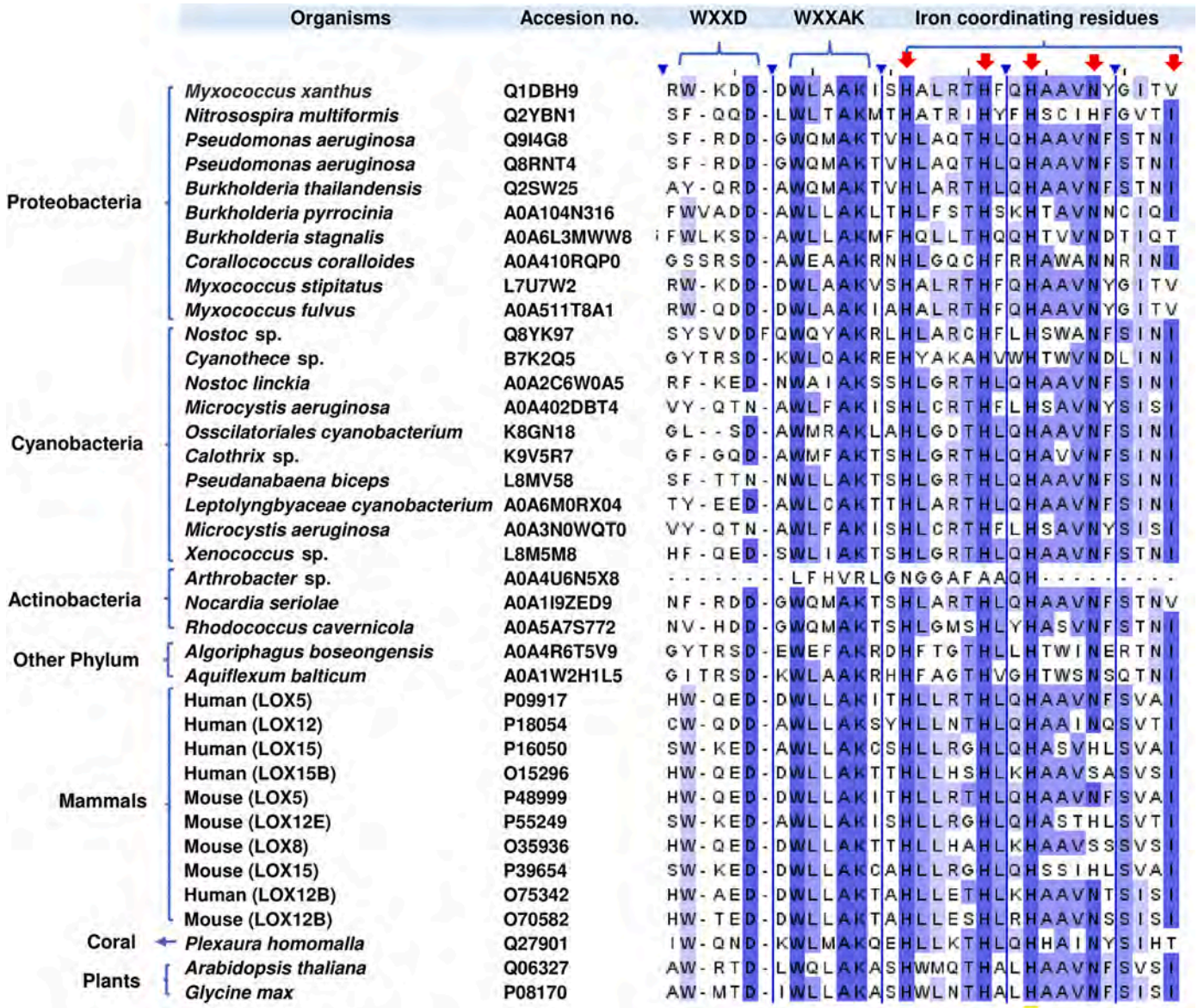

Fig. 6. Multiple sequence alignment of lipoxygenases showing the characteristic lipoxygenase WXXD and WXXAK motifs, and the iron ligand coordinating residues (three histidines, one asparagine/histidine, and the C-terminal residue, indicated by red arrows). Accession numbers of the sequences (UniProt ID) are indicated in the picture. The sequences are colored based on their conservation, with low to high conserved residues colored as light to dark blue. Multiple sequence alignment was conducted using MUSCLE and visualized using Jalview 2.11.1.4.. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Diversity and improvement of bacterial lipoxygenases

4.1. Genetic variability and phylogenetic analysis of bacterial lipoxygenases

LOXs from eukaryotic organisms can be recognized by the presence of two highly conserved sequence motifs, known as the WXXD and WXXAK motifs. The role of these motifs in the function of the enzyme is not clear yet (Ruminska et al., 2019). In bacterial LOXs, the WXXD motif is less conserved, as tryptophan (W) can be replaced by tyrosine (Y), phenylalanine (F), serine (S), valine (V) or isoleucine (I). On the other hand, the WXXAK motif seems to be conserved among all LOXs (Fig. 6). In addition to those motifs, five residues coordinate with the iron atom, consisting of three histidines (H), one asparagine (N) or histidine (H), and the C-terminal residue, which is usually isoleucine (I). Among bacterial LOXs, the three histidines are highly conserved. However, in some cases, the fourth residue, asparagine, is replaced by histidine or serine (S) and the C-terminal residue can either be isoleucine, valine or

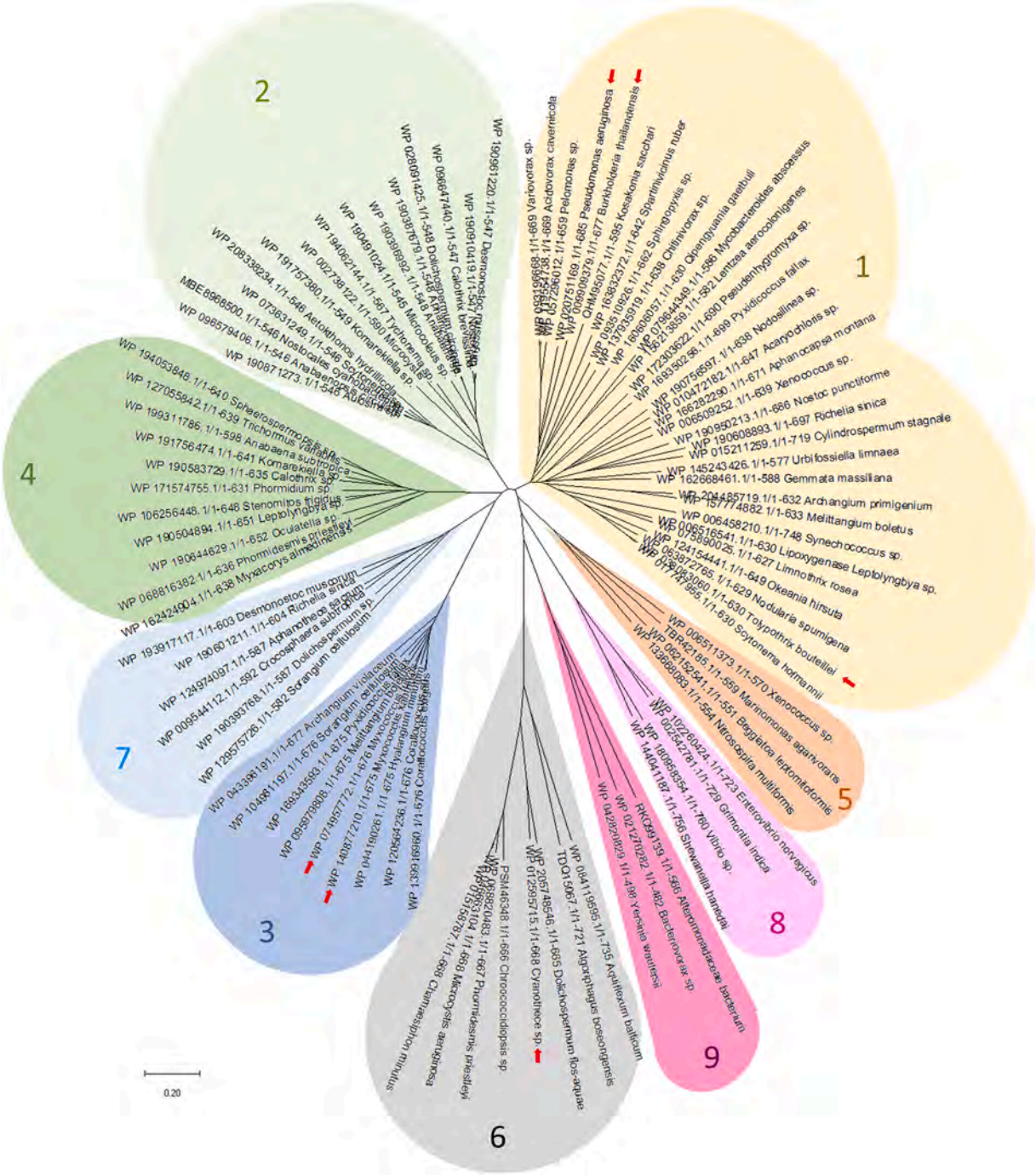

Fig. 7. Phylogenetic analysis of selected bacterial LOXs from each cluster (indicated by numbers). The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method. This analysis included 92 amino acid sequences. Evolutionary analyses and phylogenetic tree development were conducted in MEGA X. The accession number and the length of the sequences used are indicated in the figure. The characterized LOXs are indicated by red arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

threonine (T). The carboxylate group of the C-terminal residue is responsible for iron coordination, therefore this residue may not have to be conserved. Due to their highly conserved properties, the WXXAK motif and the three histidine residues coordinating the iron were used for the screening of bacterial LOXs in the following clustering and phylogenetic studies.

LOXs are produced by various bacteria from different phyla, i.e. Proteobacteria, Cyanobacteria, Actinobacteria, and others. A previous review paper on bacterial LOXs from 2013 reported 38 bacterial genes encoding for LOXs (Hansen et al., 2013). Taking a clustering based phylogenetic approach on 29 sequences of bacterial LOXs, it was found that bacterial LOXs were divided into three groups. Cyanobacteria LOXs were grouped together, while Proteobacteria LOXs were split into two groups indicating that the latter phylum has a broader sequence diversity (Hansen et al., 2013). With the huge progress in the genomics and proteomics fields, >4700 sequences from prokaryotes are currently annotated as LOX or predicted to have a LOX domain. Despite the rapidly growing number of LOX sequences from bacteria, no further clustering of bacterial LOXs has been conducted. Therefore, the clustering and phylogenetic analysis of currently known bacterial LOXs is presented in this paper.

In total, 613 bacterial sequences identified using the identical protein group resource at NCBI were used for the clustering analysis. The identical protein group resource contains only a single entry for each protein sequence found in several sources at NCBI, thereby providing a smaller dataset to work with while still representing the diversity of the bacterial sequences. Clustering analysis was conducted using CLANS (Frickey and Lupas, 2004). From this clustering analysis, ten clusters were obtained. After screening for the presence of the WXXAK motif and the three iron-coordinating histidines, one cluster containing two sequences was removed from the dataset. The remaining nine clusters were analyzed and used for phylogenetic studies.

A phylogenetic tree was constructed of 92 selected LOX sequences. For each cluster, one sequence was selected from each genus present in the cluster (Fig. 7). The phylogenetic tree was obtained using the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkendl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair by the pairwise deletion option. There were a total of 1084 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. The phylogenetic tree showed that each different cluster was found as a separate branch, hinting at their different characteristics.

The previous phylogenetic study that was conducted on 29 sequences, reported 3 groups of bacterial LOXs (Hansen et al., 2013). In line with their results, we observed that *P. aeruginosa, B. thailandensis* and *Variovorax* sp. LOXs belong to the same cluster (Cluster 1). However, *Nitrosospira multiformis* and *M. xanthus* LOXs, which were previously reported in the same group, appeared in a different cluster in our analysis (Cluster 5, and 3, respectively). Similarly, *Anabaena* sp., *Leptolyngbya* sp., and *Cyanothece* sp. LOXs were previously reported to belong to the same group, while in the present study they appeared in a different cluster (Cluster 2, 4, and 6, respectively). The difference in clustering results between our and the previous phylogenetic study can be explained by the limited number of sequences used in the previous study, as this could lead to some branches being regarded as one group.

The number of sequences present and characteristics of each cluster are presented in Table 2. The clusters are numbered from the most populated to the least. The 3D-structures of bacterial LOXs from each cluster were also predicted and modeled using Alphafold2 (Jumper et al., 2021) (Fig. 8). Cluster 1 consists of LOXs from Proteobacteria, Actinobacteria and Cyanobacteria. In this cluster, the fourth iron-coordinating residue is replaced by asparagine and the C-terminal residue is isoleucine. The Coffa site is occupied by alanine, which suggests LOXs in this cluster will display *S*-enantioselectivity. The bacterial LOXs

Table 2
Characteristics of the 9 identified bacterial LOX clusters.

Cluster	Number of Sequences	Group Member	WXXAK Motif	Iron coordinating residues*					Specificity Determinants**			
				H377	H382	H555	N559	I685	CS A402	BD 1 F353	BD 2 I593	SD IM418-9
1	282	Proteobacteria, Cyanobacteria, Actinobacteria	W-XX-AK	H	H	H	N	I	A	G/ A/ S V/ E/ N M	V/ T L/ I/ M	VA/VM/LV LL/ IL LF/ IF/ MF
2	115	Cyanobacteria	W-XX-AK	H	H	H	N	A/V/T I/M	G/ A/ L	G/A/S	T L/ I/ W	TM/ LL/ IM LF/ FM
3	77	Proteobacteria	W-LA-AK	H	H	H	N	V/ I	A	G	V/ I	LI/ FI
4	47	Cyanobacteria	W-XX-AK	H	H	H	N	I	G	A/ V/ I	L	LM/ LL
5	28	Proteobacteria	W-XX-AK	H	H	H	S/ N	N	P/ V	T/ Y	E/ V/ T	VY/ TY/ IY
6	19	Bacteroidetes, Cyanobacteria	W-LQ-AK	H	H	H	N/ H	S/V	G/ A	G/ F	A/ V M/ W	AC/ NG/ LE
7	13	Cyanobacteria, Proteobacteria	W-XX-AK	H	H	H	N	I	A	A/ V/ I	L	TG/ TS
8	4	Cyanobacteria, Proteobacteria	W-XX-AK	H	H	H	N/ H	A/V	V/ H/ F	G/ A/ V	F/ Y	QL/ LF/ MF
9	4	Proteobacteria	W-EL-AK	H	H	H	H	F/T/Y	A/ V	A/ V/ I	L/ M	TV/ TI YL/WM

Residue size: XS, S, M, L, XL

Note: *residues that aligned to iron coordinating residues of *P. aeruginosa* LOX. **residue(s) that aligned to regio- and enantioselectivity determinants of Human ALOX15. CS: Coffa site, BD 1: Borngräber 1 determinant, BD2: Borngräber 2 determinant, SD: Sloane Determinant. The specificity determinants are colored based on the size of the corresponding amino acid, extra-small (XS) with a volume of 60–75 Å^3, small (S) with a volume of 76–100 Å^3, medium (M) with a volume of 101–140 Å^3, large (L) with a volume of 140–180 Å^3, and extra-large (XL) with a volume of >181 Å^3, displayed as light to dark blue. Multiple sequence alignment was conducted using MUSCLE.

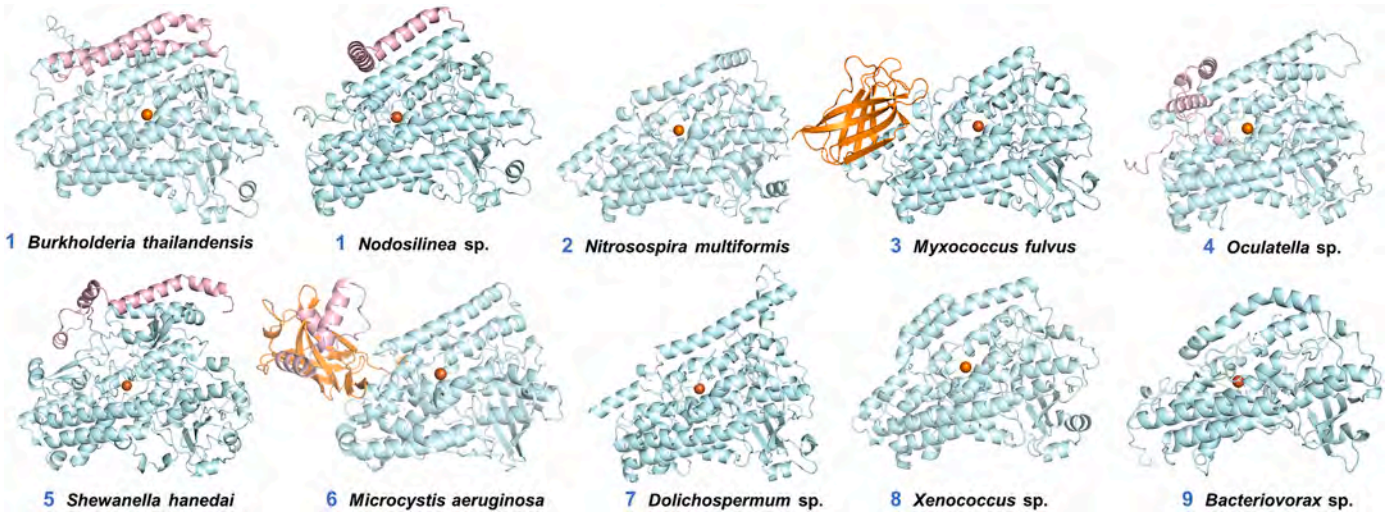

Fig. 8. Predicted structure of bacterial LOXs from each cluster. The bacterial LOXs' structures were modeled using Alphaphold2 using the casp14 settings. The number in front of the bacterial species name indicates the cluster number. The catalytic non-heme iron ion is shown as an orange sphere, the N-terminal helical extension is shown in light pink, the N-terminal β-barrel PLAT domain is shown in orange, and the C-terminal catalytic domain is shown in light cyan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from this cluster of which the enantioselectivity is known are *P. aeruginosa* PAO1 and *P. aeruginosa* BBE LOXs, and they indeed show a 13*S*-selectivity on LA (Banthiya et al., 2016; Lu et al., 2013b). The members of this cluster display quite a lot of variation in the residues forming their triad determinants, with some containing large residues (*Pelomonas* sp., *Melittangium boletus* LOXs), some containing a combination of large and small residues (*Nodosilinea* sp., *Aphanocapsa montana, Acaryochloris* sp., *Urbifossiella limnaea*, *Gemmata massiliana* and *Pseudenhygromyxa* sp. LOXs) and some also containing a combination of negatively charged residues in their BD1 and relatively bulky residues at the other sites (*P. aeruginosa* LOX and *B. thailandensis* LOX). Based on their regioselectivity, *P. aeruginosa* LOXs have shown 13-, 15- and 17-LOX activity (hydroperoxidation close to methyl end) towards LA, AA/EPA and DHA respectively (Banthiya et al., 2016; Lu et al., 2013a), confirming the effect of bulky residues present in their binding pocket. From the predicted 3D-structures, the structures of *B. thailandensis* and *Nodosilinea* sp. LOXs are similar to the known LOX structure of *P. aeruginosa* (Fig. 8). The structures contain only the C-terminal catalytic domain with an N-terminal extension consisting of two α-helices, however, the orientation of one of the α-helices of the N-terminal extension in *Nodosilinea* sp. LOXs is rotated ~90° compared to the corresponding *P. aeruginosa* LOX structure. Moreover, the helical extension in *Nodosilinea* sp. LOXs is shorter than those in *P. aeruginosa* and *B. thailandensis* LOXs. Based on the multiple sequence alignment of all members of cluster 1, the putative N-terminal helical extension can be found throughout the cluster.

In cluster 2, which consists of Cyanobacterial LOXs, the C-terminus of the enzyme is quite variable and can consist of isoleucine, valine, threonine, methionine or alanine. The Coffa site in this cluster does not only consist of alanine and glycine, but can also be filled by the bulkier leucine. This may influence the enantioselectivity of the enzymes in this cluster, although further investigation is required to understand its effect. The BD1 is always occupied by a small residue, such as glycine, alanine, or serine, which might facilitate deeper penetration of the substrate into the binding pocket. However, there is no bacterial LOX in cluster 2 that has been characterized so far. The Alphafold2 modeled structure of *Nitrosospira multiformis* from cluster 2 shows that this LOX contains only the catalytic domain (Fig. 8).

In cluster 3, which consists of Proteobacterial LOXs, the Coffa site is formed by alanine, suggesting an *S*-enantioselectivity, while the BD1 is always formed by glycine and the BD2 is occupied by isoleucine or valine. The combination of small and medium-sized residues, such as glycine and valine, in the Borngräber determinants might increase the space at the bottom of the binding pocket, and thereby facilitate a deeper entry of the substrate into the binding pocket and thus activity towards longer fatty acid substrates. As discussed above, this would result in a hydroperoxidation further away from the methyl end. The characterized bacterial LOX from cluster 3 is *M. fulvus* LOX, which indeed displays the characteristics described above, i.e. EPA-12*S*-LOX and DHA-14*S*-LOX activity (Kutzner et al., 2020). The predicted structure of *M. fulvus* LOX shows that the enzyme contains both the N-terminal β-barrel domain and the C-terminal catalytic domain, as found in plant and animal LOXs. Based on the multiple sequence alignment among all members of cluster 3, the N-terminal β-barrel domain is present throughout the cluster. This observation also confirms the annotation by InterPro (UNIPROT) of the LOX enzymes from *Myxococcus xanthus* DK 1622, *Corallococcus exiguous*, *Myxococcus fulvus*, and *Myxococcus stipitatus* DSM 14675.

Cluster 4, which consists of Cyanobacterial LOXs, may display *R*-enantioselectivity since the Coffa site is occupied by glycine. Cluster 5 consists of Proteobacterial LOXs from organisms from the genera Enterovibrio, Vibrio, Shewanella and Grimontia. In this cluster, the fourth iron-coordinating residue can be either asparagine or serine, while the C-terminal residue is always asparagine. Unlike the common A/G switch found in other LOXs, the Coffa site is occupied by valine/proline in this cluster. The predicted structures from cluster 4 (*Oculatella* sp. LOX) and 5 (*Shewanella hanedai* LOX) show that both consist of the C-terminal catalytic domain and two helical extensions (Fig. 8). However, the orientation of one or two of the helical extensions is different from that found in the structure of *P. aeruginosa* LOX from cluster 1. The confidence score of the N-terminal extension from those LOXs is lower than the C-terminal catalytic domain, suggesting that the N-terminal helical extension might be a flexible region, the function of which needs to be further investigated.

Cluster 6 consists of LOXs from Cyanobacteria and Bacteriodetes. Some members of this cluster have two small residues (glycine, alanine or valine) at their Borngräber determinants, i.e. LOXs from *Microcystis aeruginosa, Chamaesiphon minutus*, and *Phormidesmis priestleyi*, which may generate more space at the bottom of the binding pocket. The characterized bacterial LOX from this cluster is *Cyanothece* sp. PCC 8801 LOX that shows LA-9*R*-LOX activity (Newie et al., 2016). In this enzyme, the presence of glycine in its Coffa site results in *R*-enantioselectivity and

the presence of two small residues in its specificity determinants (glycine and valine) enables the substrate to slide deeper into the binding pocket leading to the addition of a hydroperoxide group at the side of the conjugated diene further away from the methyl end. The predicted structure of *Microcystis aeruginosa* LOX shows similarity with the known structure of *Cyanothece* sp. LOX, which has a C-terminal catalytic domain, a N-terminal β-barrel domain and a N-terminal helical extension (Fig. 8). Both cluster 7 and 8 contain LOXs from Cyanobacteria and Proteobacteria, while cluster 9 contains only LOXs from Proteobacteria. The second residue of the SD in cluster 7 is occupied by a small residue, glycine or serine, instead of methionine, while in cluster 8 the BD1 is occupied by a small residue: alanine, valine, or glycine. Unlike the other clusters, the Coffa site in cluster 8 and 9 is filled with a bulkier residue, i.e. phenylalanine or histidine. The predicted structures of bacterial LOXs from cluster 7 (*Dolichospermum* sp. LOX), cluster 8 (*Xenococcus* sp. LOX) and cluster 9 (*Bacteriovorax* sp. LOX) show that these enzyme only contain the catalytic domain (Fig. 8).

4.2. Currently characterized bacterial LOXs

A few bacterial LOXs have been biochemically characterized and display different optimum conditions (Table 3). *P. aeruginosa* 42A2 LOX displays an optimum activity at 25–30 °C, is active up to 45 °C, less active above 50 °C and completely inactive at 70 °C (Busquets et al., 2004; Vidal-Mas et al., 2005). A similar optimal temperature of 25–30 °C is observed for LOXs from *P. aeruginosa* PAO1 (Banthiya et al., 2016), *P. aeruginosa* BBE (Lu et al., 2013b), *B. thailandensis* E264 and *M. xanthus* DK 1622 (Qian et al., 2017). On the other hand, *M. fulvus* LOX shows its highest activity at very low temperatures of 5–10 °C (Goloshchapova et al., 2018). Although most characterized bacterial LOXs display an optimum temperature at relatively low temperature, one bacterial LOX purified from *P. aeruginosa* PR3 displays a significantly higher optimum temperature of 60 °C and thermostability by maintaining approximately 80% of its activity after incubation for 3 h at 60 °C (Bae et al., 2010).

Most LOXs from eukaryotic organisms are optimally active at a neutral pH of around 6.5–7.5 (Koch et al., 1992; Kuhn et al., 1993). However, some bacterial LOXs were reported to have optimal activity at extreme pH values. *M. xanthus* DK 1622 produces an acidic 11*S*-LOX, which shows an optimal activity at pH 3, although the enzyme is more stable at pH 6 (Qian et al., 2017). On the other hand, *M. fulvus* LOX-1 acts as an alkaline 12*S*-LOX with an optimum activity at pH 9 (Goloshchapova et al., 2018). The resistance and stability of some bacterial LOXs towards extreme conditions can become important features for their application as industrial biocatalysts.

Bacterial LOXs differ regarding their substrate preference. The enzymes from *B. thailandensis* E264, *Rivularia* sp. PCC 7116, *Calothrix* sp. HK-06, *Tolypothrix bouteillei, Sphingopyxis macrogoltabida* and *Pseudomonas* sp. 42A2 were reported to have the highest activity towards LA (An et al., 2015; Busquets et al., 2004; Kim et al., 2022; Qi et al., 2020). The activity towards LA has also been reported for LOX from *Nostoc* sp. PCC 7120 (Andreou et al., 2008) and *P. aeruginosa* strains PR3 and BBE (Bae et al., 2010; Lu et al., 2013b). *Archangium violaceum* and *Endozoicomonas numazuensis* LOX displayed the highest activity towards AA (Kim et al., 2021; Lee et al., 2022). LOX from *P. aeruginosa* PAO1 showed the highest activity towards dihomo-γ-linolenic acid (DGLA; C20:3 Δ8,11,14) followed by DHA (Banthiya et al., 2016). LOXs from *P. aeruginosa* PAO1 and *M. xanthus* DK 1622 were also reported to have high activity towards AA (Banthiya et al., 2016; Qian et al., 2017), while LOX from *M. fulvus* was reported to be most active with EPA (Goloshchapova et al., 2018). Among all the characterized bacterial LOXs, those from *Rivularia* sp. PCC 7116 and *Calothrix* sp. HK-06 have the highest activities reported so far (Qi et al., 2020). The wide diversity in substrate preference of bacterial LOXs raises the opportunity to use these enzymes with many substrates, e.g. plant oil hydrolysates which mostly contain oleic acid LA and ALA, as well as algal oil hydrolysates, which contain a broad range of fatty acids including EPA and DHA. Bacterial LOXs catalyze dioxygenations of fatty acids in a regioselective way (Fig. 9). This may lead to a number of interesting applications in the chemical and food industries, for example in the production of oleochemicals and flavor/aroma compounds.

4.3. Enzyme improvement approaches

The wide range of substrate specificities and regio−/enantioselectivities of bacterial LOXs provide them with remarkable characteristics for potential applications. However, most characterized bacterial LOXs display rather poor thermostability and some of them also display low activity compared to LOXs from other organisms, e.g. soybean. For industrial applications, LOXs with an improved activity and stability are required. In the area of biocatalysis, enzyme improvement can be achieved through several approaches, i.e. (1) rational design through site-directed mutagenesis or structure-guided engineering, (2) directed evolution through error prone PCR, DNA shuffling or saturation mutagenesis, (3) semi-rational approaches through region-specific random mutagenesis or site-saturation mutagenesis, and (4) in silico computational protein design. These enzyme engineering approaches can help to improve the enzymatic properties of bacterial LOXs for industrial applications. Some reported approaches to improve bacterial LOXs are summarized in Table 4.

Improvement of bacterial LOXs was mostly conducted by changing the regio- and enantio-selectivity of the enzyme depending on the product of interest. Changing the enzyme selectivity from 12*S*- to almost exclusively 15*S*-regioselective dioxygenation was reported by mutation of Thr397Tyr at the BD1 of *M. xanthus* LOX (An et al., 2018a). An opposite change in selectivity, from 15*S*- to 12*S*-regioselective dioxygenation, was reported for *Archangium violaceum* LOX by a Leu429Ala + Leu430Ala double mutation (Lee et al., 2022). Changing the enzyme specificity from 9*R*- to almost exclusively 13*S*-dioxygenation or vice versa was achieved in some bacterial LOXs by exchanging the Coffa site residue for a bulkier amino acid residue (Andreou et al., 2008; Newie et al., 2016; Yi et al., 2020). Improving the enzyme activity by enlarging the oxygen channel through structure-guided engineering (Ala324Gly/Ser392Gly double substitution) has been reported recently (Qi et al., 2021).

Improving the thermostability of the enzyme is one of the key factors for successful application of bacterial LOXs in industry. Several approaches have been introduced for thermostability enhancement. A site-directed mutagenesis approach based on computer-aided rational design was followed to successfully increase the enzyme thermostability and specific activity of *Anabeana* LOX by substituting Val40 or Val421 and both these residues by Ala (Diao et al., 2016). Enhancing the enzyme stability was also achieved by modifying the highly flexible regions of *P. aeruginosa* LOX. Modification of residues 20–49 and residues 201–206 resulted in a significant enhancement of the thermostability (Lu et al., 2014). Directed evolution by error-prone PCR and DNA shuffling also gave promising results to increase the LOX thermostability (Guo et al., 2014). The production of a fusion protein by combining self-assembling amphipathic peptides (SAPs) at the N-terminus of *P. aeruginosa* LOX was reported to improve the thermostability and specific activity of the enzyme (Lu et al., 2013a). The SAPs contain unique sequences with alternating hydrophobic and hydrophilic residues that can spontaneously assemble into ordered nanostructures.

5. Potential applications of bacterial lipoxygenases

5.1. LOX as starting biocatalyst for the production of oleochemicals

The regioselective hydroperoxidation of fatty acids catalyzed by LOXs has a number of interesting applications for the production of oleochemicals. The fatty acid hydroperoxides produced by LOXs can be used as intermediates for the preparation of different oleochemicals. The chain length of the degradation products depends on the position of the

Table 3
The reported activity and optimum conditions of biochemically characterized bacterial LOXs.

Enzyme Source	Cluster	Fatty Acid Substrate	Relative activity (%)	Specific activity (U. mg^{-1})	Oxygenation Specificity	Optimum pH	Optimum Temperature (°C)	Ref.
Pseudomonas sp. 42A2	1	OA	46	NR	10	8.5–9	25	(Busquets et al., 2004)
		LA	100		NR	NR	NR	
		ALA	60					
		AA	1					
Pseudomonas aeruginosa PR3	1	LA	100	NR	9	6	60	(Bae et al., 2010)
					13			
		GLA	120		NR	NR	NR	
Pseudomonas aeruginosa PAO1	1	LA	50	NR	13*S*	NR	25	(Banthiya et al., 2016)
		ALA	11		13	NR	NR	
		GLA	11		13			
		AA	100		15			
		EPA	6		15			
		DGLA	428		15			
		DHA	367		17			
Pseudomonas aeruginosa BBE	1	LA	–	28.3	13*S*	7.5	25–30	(Lu et al., 2013b)
Burkholderia thailandensis E264	1	LA	100	26.4	13	7.5	25	(An et al., 2015)
		ALA	40	10.5	NR	NR	NR	
		GLA	11	2.9				
		AA	86	22.6				
Rivularia sp. PCC 7116	1	LA	100	68.8	13*S*	8.5	30	
		ALA	91	63.2	NR	NR	NR	
		GLA	84	58				
		AA	27	18.8				
Calothrix sp. HK-06	1	LA	100	73.1	13*S*	8	25	(Qi et al., 2020)
		ALA	43	31.7	NR	NR	NR	
		GLA	55	40.4				
		AA	65	47.8				
Tolypothrix bouteillei VB521301	1	LA	100	10.1	13*S*	8	30	
		ALA	18	1.8	NR	NR	NR	
		GLA	18	1.8				
		AA	29	2.9				
Sphingopyxis macrogoltabida	1	LA	100	0.21	9*S*	NR	NR	(Kim et al., 2022; Oh et al., 2022)
		ALA	65	0.14	13*S*			
		GLA	53	0.11	9*S*			
		AA	75	0.16	9*S*	8.5	35	
		EPA	52	0.11	9*S*	NR	NR	
		DPA	65	0.14	11*S*			
		DHA	53	0.11	11*S*	8.5	35	
		9*S*-HETE	15	0.03	15*S*	NR	NR	
		9*S*-HEPE	12	0.025	15*S*			
		11*S*-HDPE	38	0.08	17*S*			
		11*S*-HDHE	30	0.06	17*S*			
Endozoicomonas numazuensis	1	AA	100	24.7	5*S*,12*S*	NR	NR	(Kim et al., 2021)
		EPA	62	15.3	5*S*,12*S*			
		DPA	35	8.6	7*S*,14*S*			
		DHA	52	12.9	7*S*,14*S*			
Myxococcus xanthus	3	LA	100	24.0	13*S*	NR	NR	(An et al., 2018a)
		ALA	60	14.4	13*S*			
		GLA	52	12.5	13*S*			
		AA	64	15.3	12*S*			
		EPA	37	8.9	12*S*			
		DHA	31	7.5	14*S*			
Myxococcus xanthus DK 1622	3	LA	100	NR	NR	3	30	(Qian et al., 2017)
		AA	91			NR	NR	
Myxococcus fulvus	3	ALA	23	NR	13	NR	NR	(Goloshchapova et al., 2018)
		AA	50		12*S*			
		EPA	100		12*S*	9	5–10	
		DHA	52		14*S*	NR	NR	
Archangium violaceum	3	AA	100	0.05	5*S*, 15*S*	NR	NR	(Lee et al., 2022)
		EPA	62	0.03	5*S*, 15*S*			
		DPA	13	0.026	7*S*, 17*S*			
		DHA	52	0.006	7*S*, 17*S*			
Cyanothece sp. PCC 8801	6	LA	NR	NR	9*R*	NR	NR	(Newie et al., 2016)
		ALA			9*R*			
		AA			12*R* *			
					11*R*			
		EPA			11			
					14*			
Nostoc sp. PCC 7120	6	LA	NR	NR	9*R**	NR	NR	(Andreou et al., 2008; Lang et al., 2008)
					13*S*			
		ALA			9*R**			

(*continued on next page*)

Table 3 (*continued*)

Enzyme Source	Cluster	Fatty Acid Substrate	Relative activity (%)	Specific activity (U. mg^{-1})	Oxygenation Specificity	Optimum pH	Optimum Temperature (°C)	Ref.
					13*S*			
		AA			11*R**			
					15*S*			
		LA			9*R*			
Acaryochloris marina	6	ALA	NR	NR	12*R*	NR	NR	(Gao et al., 2010)
		EPA			11*R*			

Note: The relative activities given are relative only for that specific enzyme. U: one unit of LOX activity is defined as the amount of enzyme required to produce 1 μmol hydroperoxy fatty acid per min under specific condition. NR: not reported, *predominant product. The reported enantioselectivity of the enzymes are indicated.

peroxide group on the fatty acid. Using specific LOX and/or fatty acids as substrate, various sizes of degradation products can be generated. Bacterial LOXs provide a wide range of substrate specificities and regioselectivities (Fig. 9) that potentially allow the generation of various hydroperoxy derivatives.

Fatty acid hydroperoxides produced by LOXs can be converted into hemiacetals and the formed hemiacetals then dissociate to yield aldehydes, which can serve as precursors to shorter-chain polymer building blocks such as diacids, epoxides, diols or ω-hydroxy fatty acids. Moreover, hydroperoxide fatty acid derivatives may be used as multifunctional surfactants in food and cosmetics production, since they allow both bleaching and washing performances at a lower temperature (Zhang et al., 2012). Hydroperoxy fatty acids can also be reduced to hydroxy fatty acids, which are of interest for application as chemical intermediates (Song et al., 2013). Hydroxy fatty acids have been utilized as starting materials for the production of lubricants (Mutlu and Meier, 2010), surfactants (Hu et al., 2014), plastics (Ashby et al., 2016), and biobased polymers (Liu et al., 2012). Hydroxy fatty acids can also be applied as additives in paint and coating materials because their reactivity is enhanced compared to the non-hydroxylated fatty acids (Hou, 2009). The industrial use of LOX-derived hydroperoxy and hydroxy fatty acids as a sustainable, biobased resource and environmentally friendly technology for those applications might contribute to generating a more sustainable world by replacing the use of petroleum oil.

5.2. LOX for the production of flavor/aroma compounds

LOXs are of importance in the food industry, since the fatty acid hydroperoxides that they form are involved in the formation of pleasant flavors, called green leaf flavors, during food technology processes. For example, 2*E*-hexenol displays fruity flavors and can be used as a flavor enhancer for alcoholic beverages, while 3*Z*-hexenol presents a powerful green grass odor (Gigot et al., 2010). Green leaf volatiles are generally aldehydes and alcohols produced by combined action of LOX, hydroperoxide lyase and alcohol dehydrogenase (Fig. 10). Short-chain volatile aldehydes such as hexanal and 3*Z*-nonenal can be generated by hydroperoxide lyase-catalyzed splitting of fatty acid hydroperoxides. Hydroperoxide lyase cleaves fatty acid hydroperoxides into 6- or 9-carbon volatile aldehydes depending on the specificity of the enzyme (Casey and Hughes, 2004). These aldehydes can then be further converted into alcohols by alcohol dehydrogenase. Because of their specificity, bacterial LOXs have a promising application for the production of these flavor/aroma compounds with high purity.

5.3. LOX for the production of signaling compounds and lipid mediators

LOXs have gained attention as a potential starting biocatalyst for the production of various signaling compounds and lipid mediators that could potentially be applied not only for clinical uses but also for agricultural uses (Joo and Oh, 2012). Pro-resolving lipid mediators such as lipoxins, resolvins, protectin and maresin, can be produced by further conversion of LOX-derived hydroperoxy fatty acids. In addition to producing hydroperoxy fatty acids through dioxygenation reactions, some LOXs have also been reported to catalyze subsequent reactions involving dehydration or isomerization of the hydroperoxides to produce epoxy fatty acids or hydroxy epoxy fatty acids respectively (An et al., 2018b; Maas and Brash, 1983; Smyrniotis et al., 2014; Yu et al., 2003; Zheng and Brash, 2010). These epoxy intermediates can be transformed further into hepoxilins, trioxilins, leukotrienes and lipoxins (An et al., 2021, 2018b; Haeggström and Funk, 2011).

Lipoxins (trihydroxy-eicosapolyenoic acids with a conjugated tetraene) can be produced from AA by combined actions of lipoxygenases (either the 15- and 5-LOX or the 5- and 12-LOX) and peroxidases (Samuelsson et al., 1987). Lipoxins have also been reported to act as anti-inflammatory, organ-protective, and anti-fibrotic mediators (Fu et al., 2020; Kurtoğlu et al., 2019; Roach et al., 2014; Zhou et al., 2011). D-series resolvins, protectin and maresin can be synthesized from DHA by involving LOX to facilitate molecular oxygen insertion specifically at carbon atom position 17 or 14, while E-series resolvins can be synthesized from EPA by the combined action of cyclooxygenase (COX-2) or P450 and LOX (Serhan, 2014; Serhan and Petasis, 2011). Plant jasmonic acids which are produced from ALA by the combined action of LOX, allene oxide synthase, and allene oxide cyclase can be used for pest and stress control in plants, because they can act as a signaling molecules that induce the natural plant defense mechanisms in response to certain stressors (insects, mechanical stress, water deficiency) without inhibiting plant growth (Montillet et al., 2005).

5.4. LOX as food additives

LOXs can also be applied as food additives. For example, LOXs have been applied for the co-oxidation of carotenoid pigments in cereal flour to give a brighter colour of flour (Hayward et al., 2017). LOXs have also been used in the cheese industry during whey decolorization treatment to destruct the carotenoid colorant annatto (Kang et al., 2010). Furthermore, LOXs can generate peroxides that are able to oxidize the sulfhydryl groups of wheat protein, forming disulfide bonds, strengthening the gluten and giving a cross-linking effect on the flour proteins, which in turn affects the structure and rheology of the dough (Permyakova and Trufanov, 2011).

6. Conclusion

Bacterial LOXs have great biotechnological potential. They can be easily produced in heterologous hosts and therefore are readily modified by protein engineering, are active towards a wide range of fatty acids, display high regio- and enantioselectivity, and some of them are active at extreme conditions, i.e. pH values and temperature. These advantages stimulate their exploitation as green alternatives in a wide range of applications, from the production of oleochemicals to the development of flavor compounds, the synthesis of various signaling compounds and use as food additives. However, only a few bacterial LOXs have been biochemically characterized so far. In this study, clustering and phylogenetic analyses revealed that there are nine clusters of phylogenetically related bacterial LOXs, characterized by specific residues in their Coffa site and other specificity determinants. These differences in residues

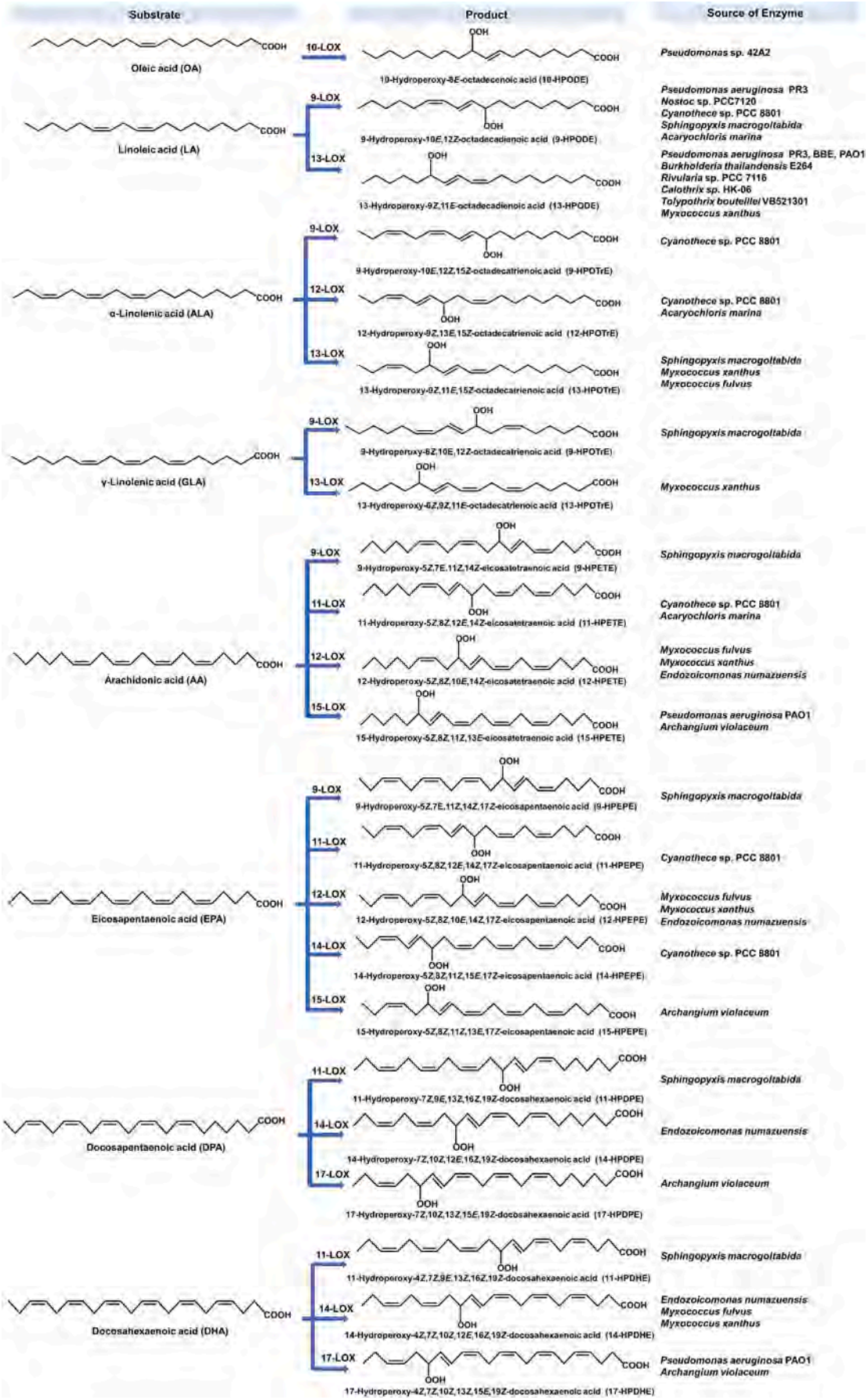

Fig. 9. Reported regioselective dioxygenations of fatty acids catalyzed by bacterial LOXs.

Table 4
Improvement of bacterial lipoxygenases by protein engineering.

Enzyme Alteration	Source	Approach	Modification	Result	Ref.
Regio- and/or enantioselectivity	*M. xanthus*	Rational design	Substitution Thr397Tyr (BD1)	Change of enzyme specificity from 12*S*- to almost exclusively 15*S*-dioxygenation	(An et al., 2018a)
	Cyanothece sp.	Rational design	Substitution Gly401Ala (CS)	Change of enzyme specificity from 9*R*- to almost exclusively 13*S*-dioxygenation	(Newie et al., 2016)
	Nostoc sp. PCC 7120	Rational design	Substitution Ala162Val and Ala162Ile (CS)	Change of enzyme specificity from 9*R*- to almost exclusively 13*S*-dioxygenation	(Andreou et al., 2008)
	Oscillatoria nigro-iridis PCC 7112	Rational design	Substitution Ala296Gly (CS)	Change of enzyme specificity from 13*S*- to 9*R*-dioxygenation	(Yi et al., 2020)
	Archangium violaceum	Rational design by structure-based engineering	Substitution Leu429Ala/ Leu430Ala	Change of enzyme specificity from AA 15*S*- to 12*S*-dioxygenation	(Lee et al., 2022)
Activity	*Rivularia* sp. PCC 7116	Rational design by structure-guided engineering	Ala324Gly/Ser392Gly	Enlargement of oxygen channel and Increase in activity by 3 to 5-fold.	(Qi et al., 2021)
Thermostability	*Anabaena* sp. PCC 7120	Site-directed mutagenesis based on computer-aided rational design	Substitution Val421Ala Substitution Val40Ala Double Substitution Val421Ala + Val40Ala	Increase of optimal temperature by 5 °C, the half-life times increased 1.18 fold. Val421Ala, Val40Ala, and Val421Ala/Val40Ala also displayed 42, 5, and 80% increase in specific activity, respectively	(Diao et al., 2016)
	Anabaena sp. PCC 7120	Directed evolution by error prone PCR & DNA Shuffling	Substitution Asn305Asp	Increase of optimum temperature from 45 °C to 50 °C and increase of catalytic efficiency (*k*cat/*K*m) by 83%.	(Guo et al., 2014)
	Pseudomonas aeruginosa	Rational design	Deletion of the first 20 and 30 residues	Increase of half-life times at 50 °C by 1.3- and 2.1-fold, respectively. The optimum temperature increased 5 and 10 °C, respectively	(Lu et al., 2014)
			Substitutions of Gly204Pro, Gly206Pro, and Gly204Pro + Gly206Pro	Increase of half-life times at 50 °C by values ranging from 0.46- to 3.45-fold. The optimum temperature increased by 5–15 °C.	
	Pseudomonas aeruginosa	Rational design by structure-guided engineering	Fusing with self-assembling amphipathic peptides	The mutant showed 2.3- to 4.5- fold enhanced thermal stability at 50 °C. The specific activity increased by 1.0- to 2.8-fold as compared with the wild-type	(Lu et al., 2013a)

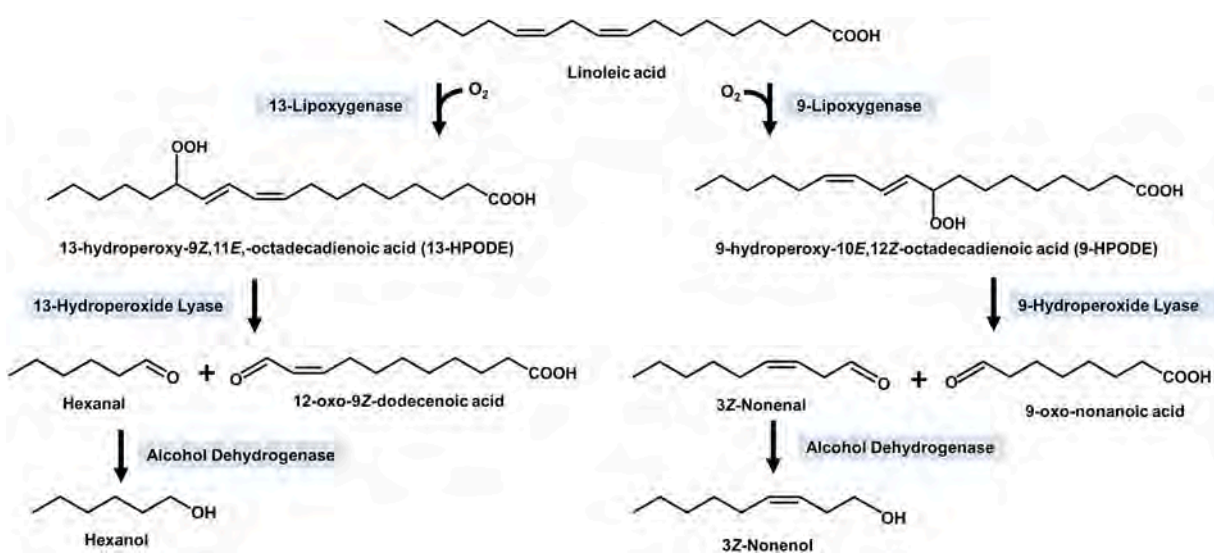

Fig. 10. Aldehydes and alcohols, flavor aromas in many fruits and vegetables, are formed by the combined action of lipoxygenase, hydroperoxide lyase and alcohol dehydrogenase.

may provide valuable information on the substrate specificity and regio−/enantioselectivity of the LOXs present in each cluster. Therefore, activity assays using various fatty acid substrates and analysis of the products need to be conducted to confirm the different characteristics of each cluster of bacterial LOXs. Although there are some reports on successful heterologous expression of bacterial LOXs, development of efficient production systems for fatty acid hydroperoxides and/or their derivatives from unsaturated fatty acids by bacterial LOXs is needed in order to exploit their potential as industrial biocatalysts.

CRediT authorship contribution statement

Ruth Chrisnasari: Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis, Investigation, Visualization. **Marie Hennebelle:** Writing – review & editing, Conceptualization, Supervision. **Jean-Paul Vincken:** Writing – review & editing, Supervision. **Willem J.H. van Berkel:** Writing – review & editing. **Tom A. Ewing:** Writing – review & editing, Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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