Microbiological Research 222 (2019) 1–13

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Multicopper oxidases: Biocatalysts in microbial pathogenesis and stress management

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ARTICLE INFO

Keywords:
Multicopper oxidases
Copper centers
Metal homeostasis
Stress management
Pathogenicity

ABSTRACT

The acquisition of metal ions such as iron, copper and manganese is essential for the survival of microorganisms as these are constituents of metalloproteins including enzymes, storage proteins, structural elements, transcription factors and antimicrobial factors in various biological processes. However, excess of these metal ions is associated with significant toxicity due to spontaneous redox cycling of ions and obstruction of normal metabolic pathways. To overcome this, microbes have developed a variety of metal regulatory systems allowing them to adapt to the changing biotic and abiotic environments. Multi-copper oxidases (MCOs) such as ceruloplasmins, ferroxidases, laccases and nitrite reductases are such regulatory systems employed by microbes to resist the toxicity of metal ions by controlling their oxidation states under aerobic conditions. MCOs help pathogens survive during an infection by evasion of the toxic environment generated by the host immune system and thus are considered necessary determinants of virulence. This review summarizes the role of MCOs in metal homeostasis under stressful conditions and the extent to which these MCOs contribute to microbial virulence within the host that might prove as an esteemed avenue for the development of novel antimicrobial therapies.

1. Introduction

Metals play a critical role in the growth and biology of every cell. They act as cofactors in various enzymatic processes and are also important components of metabolic machinery of cell. The propensity of metals to readily lose electrons and participate in a variety of redox reactions makes it a necessity for the cell to maintain internal metal homeostasis (Chandrangsu et al., 2017). In addition, metals are major players at the interface of host and pathogen. In a well known process known as nutritional immunity, host limits the availability of essential metal ions or generates toxic environment so as to restrict the survival and pathogenesis of the invading microbes (Hood and Skaar, 2012). In response, pathogens have emerged with specialized metal acquiring regulatory systems and/or efflux pumps to defend host metal scarcity or toxicity.

Multicopper oxidases (MCOs) constitute one such regulatory system whose metalloxidase activity has made them important components of metal homeostasis. They are widely distributed in nature and have unique structural, spectroscopic and functional properties. Members of this family include ceruloplasmins (ferroxidases), ascorbate oxidases, laccases and nitrite reductases (Hoegger et al., 2006). They catalyze the one electron oxidation of a variety of substrates along with four electron reduction of oxygen to water. Three different copper centers are involved in the redox reaction catalyzed by MCOs that can be distinguished by UV/Vis and electronic paramagnetic resonance (EPR) spectroscopy (Sakurai and Kataoka, 2007). These are T1 copper center (blue) which shows strong absorption at 600 nm with narrow hyperfine splittings in the EPR spectroscopy, T2 copper center (normal) showing no absorption in UV/Vis spectroscopy with normal hyperfine splittings in the EPR spectrum, T3 copper center (coupled binuclear) which shows absorbance signal at 330 nm but is not detected in the EPR spectroscopy (Fig. 1). The T2 and T3 copper centers in conjugation form the trinuclear cluster. Amino acid residues 'HCH' connect T1 copper center over 12 Å to trinuclear copper center, cytosine being the ligand for T1 Cu and histidines coordinating with two T3 copper atoms. Electrons are transferred from the site of substrate oxidation i.e. T1 copper center to trinuclear copper cluster where oxygen binds and is reduced to water via an outer-sphere two-electron transfer reaction mechanism (Bello et al., 2012). The first two-electron transfer reaction is a rate limiting step and involves the conversion of dioxygen to peroxide intermediate and the second two-electron transfer reaction catalyze the reductive cleavage of $O{-}O^{-}$ bond of peroxide intermediate to generate native intermediate. The second conversion reaction is very fast and requires the involvement of one proton which gets shuttled to highly conserved

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https://doi.org/10.1016/j.micres.2019.02.007
Received 21 November 2018; Received in revised form 3 January 2019; Accepted 14 February 2019
Available online 16 February 2019
0944-5013/

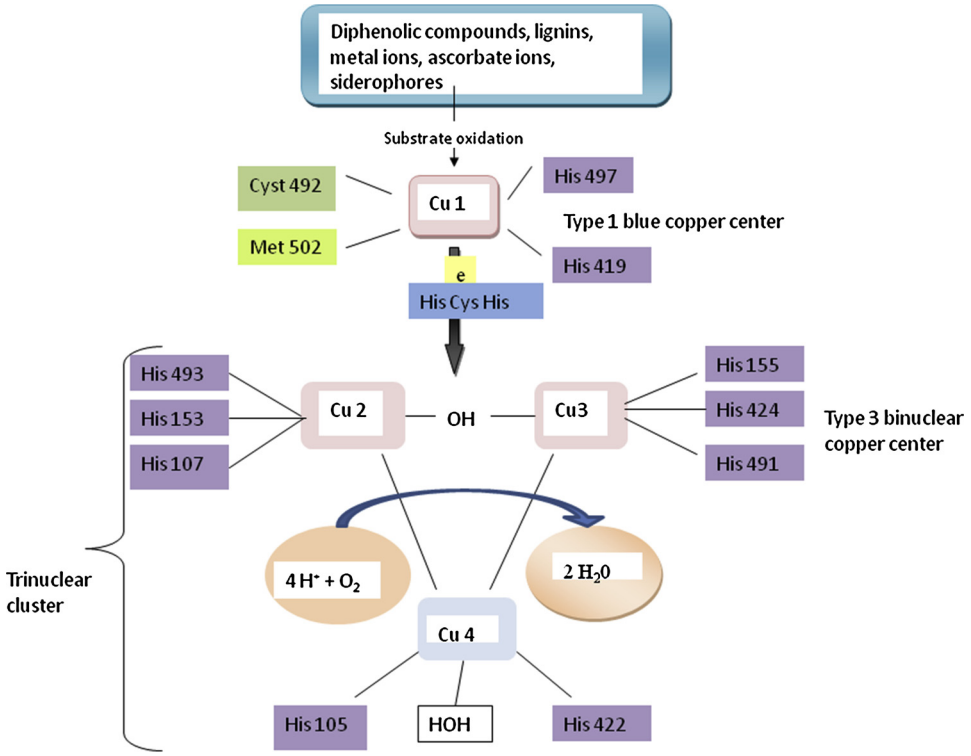

Fig. 1. Schematic representation of functional copper centers in multicopper oxidases. Type 1 copper (Cu 1) catalyzes the oxidation of substrate by the transfer of electrons via highly conserved HCH connecting motifs to the trinuclear cluster (Cu 2-Cu 3 and Cu 4) and subsequent reduction to water.

acidic residues of Glu or Asp at trinuclear copper centres for the oxygen reduction process (Serrano-Posada et al., 2015; Liu et al., 2018). A hydrophobic core site near the T1 Cu determines the substrate specificity of MCO and proper coordination of copper is essential for the conformational mobility and activity of enzyme (Bello et al., 2012, 2014).

MCOs comprise of 2, 3 or 6 homologous cupredoxin domains which are structurally present in a ß-sandwich pattern i.e. 8 strands in 2 ß sheets arranged in Greek key beta-barrel shape (Roberts et al., 2002). Ceruloplasmin is a 6-cupredoxin domain MCO, laccase and ascorbate oxidase comprise of 3 such domains whereas nitrite reductase has 2 domains. T1 copper center is present in domain 3 with a shallow depression for the binding of substrate; however trinuclear cluster is present at the interface of domains1 and 3 (Sedlak et al., 2018). Arrangement of cupredoxin domains and their interactions with copper centres are critical for the activity of MCOs (Herrera-Zúñiga et al., 2018). 3-D structures of MCOs which are available in Protein data bank has been illustrated in the table S1 of supplementary data.

Despite their structural similarity, MCOs are diverse in substrate specificity and functional roles. They oxidize lignin rich aromatic compounds, polyphenols, metal ions, ascorbate ions, siderophores and pigments. They play important roles such as spore coat resistance (Enguita et al., 2003; Sharma et al., 2007), melanin production (Hullo et al., 2009), morphogenesis (Driks, 2004) metal oxidation (Huston et al., 2002; Zhang et al., 2015) and denitrification (Lawton et al., 2013; Long et al., 2015) in bacteria; pigment formation (Langfelder et al., 2003), lignin degradation (Casadevall et al., 2009), dissimilatory nitrite reduction (Matsuoka et al., 2017) and virulence in fungi (Zhu and Williamson, 2004); iron uptake in yeasts (Stoj et al., 2006); cuticle tanning in insects (Dittmer and Kanost, 2010), lignin biosynthesis and ascorbate metabolism in plants (Turlapati et al., 2011); and iron metabolism in mammals (Harned et al., 2012). Recently, some new enzymes such as bilirubin oxidase, phenoxazinine synthase, dihydrogeodin synthase, sulochrin oxidase have been added in the MCO superfamily which participates in birilubin oxidation, biosynthesis of antibiotics and other fungal metabolites respectively (Table S2). Thus MCOs offer various functions depending on the physiological and pathological conditions of the organism.

As the structural characterization of MCOs has been well elucidated in previous studies (Hoegger et al., 2006; Sakurai and Kataoka, 2007; Lawton et al., 2009; Komori and Higuchi, 2015; Li et al., 2015; Sedlak et al., 2018), this review sets the basis to understand role of MCOs in host-pathogen interface by maintaining metal homeostasis and protection from adverse stressful environments.

2. Ceruloplasmin and ferroxidase: catalysts in iron homeostasis and virulence

Iron is essential for the growth and cellular processes of all biological systems and is thus considered a critical micronutrient for pathogenesis (Zaveckas et al., 2000). The optimum concentration of iron required for microbial survival is 10^{-8}-10^{-6} M which is much higher in comparison to the availability of free iron in the host (10^{-17} M) (Braun and Killmann, 1999). Host restricts the availability of iron as a defense mechanism against pathogens via a process known as nutritional immunity (Hood and Skaar, 2012). Also, ferrous ions under aerobic condition are toxic in nature as they cause oxidative stress in cell via the generation of reactive hydroxyl radicals (Fenton's and Haber-Weiss reaction) (Kehrer, 2000). Thus it is imperative for the pathogen to regulate balance between iron acquisition and toxicity for the successful dissemination and pathogenesis inside the host. Therefore, iron homeostasis is a complex mechanism and is regulated by a group of different proteins which contribute to its uptake, efflux, utilization and storage.

Ceruloplasmin (CP), a copper-oxidase enzyme, is responsible for iron metabolism and homeostasis in humans. The physiological role of ceruloplasmin is associated with its ferroxidase activity which catalyzes the oxidation of Fe (II) to Fe (III) ions for further mobilization to transferrin and ferritin proteins. Previously known as blue plasma amine oxidase, it is the first well characterized 6-domain multicopper oxidase, synthesized in liver and carrier of 95% of total copper found in

human plasma (Chen et al., 2004). Expression of ceruloplasmin has been manifested in other human tissues as well such as glycophosphatidylinositol (GPI)–CP in brain astrocytes and reticuloendothelial cells, hephaestin (HEPH) in intestinal enterocytes and zyklopen (ZP) in placenta. Here also, their presumed function is to act as ferroxidase and participate in iron efflux and transport (Chen et al., 2009; Prohaska, 2011; Jiang et al., 2016). Homologous to mammalian ceruloplasmin is the ferroxidase protein present in fungi and bacteria (Fig. S1). Ferroxidases form an important component of high affinity reductive iron uptake system in fungi. This system embraces the involvement of certain proteins viz. cell surface reductases for the reduction of ferric ions to ferrous ions, ferroxidases for the oxidation to ferric ions and iron permeases for the transportation of soluble ferric ions inside the cell. Such iron acquisition systems have been best characterized in *Saccharomyces cerevisiae* (de silva et al., 1996) and are widely distributed in pathogenic fungi viz. *Cryptococcus neoformans* (Jung et al., 2009), *Aspergillus fumigates* (Blatzer et al., 2011) and *Candida albicans* (Zieglar et al., 2012). In *C. neoformans*, which is a non-siderophore producing fungus, ferroxidase (Cfo1) besides playing role in iron uptake also contributes to its pathogenesis as the mutant lacking *cfo1* gene showed growth defects, reduced intracellular iron concentration and inability to utilize inorganic iron or transferring sole iron sources. This resulted in reduced colonization in brain and increased sensitivity towards copper ions and anti fungal drugs. As a whole, virulence of this pathogen was strongly attenuated by the loss of ferroxidases (Fig. 2) (Jung et al., 2009).

Ferroxidase mediated iron acquisition has also been presumed to play central role in bacterial pathogenesis (Table 1). A cytoplasmic MCO (McoL) helps in the extracellular growth of facultative pathogen *Legionella pneumophila* under either iron restricted environment or in the presence of sole iron Fe (II) source. It also acts as an important virulence factor by further extending its role in protection from toxicity of ferrous ions under aerobic environments (Huston et al., 2008). Similarly in *Pseudomonas aeruginosa*, MCO encoding ferroxidase plays role in the uptake of Fe (II) and its subsequent oxidation but only under aerobic conditions. In an interesting study conducted by de Vos et al. (2001), *P. aeruginosa* strains isolated from cystic fibrosis patients lacked siderophores which are important iron chelating virulence factors of this pathogen. In such cases, role of ferroxidase-mediated iron acquisition comes into play which operates independent of siderophores-mediated iron uptake suggesting it as a critical virulence determinant in pathogenesis. Bacterial ferroxidases have crucial role in iron uptake and metabolism only and showed no sensitivity towards toxic copper ions. In conclusion, ferroxidases have been recognized as a novel feature of microbial pathogenicity, but there are some questions remaining to be answered at molecular level such as the contribution of iron-sensing transcriptional regulators and signaling molecules for the expression of ferroxidases in iron uptake and pathogenesis.

3. Laccases: versatile catalysts in melanogenesis, stress adaptation and virulence

Laccases form the largest subclass of multicopper oxidase family as these are ubiquitous in all biological forms ranging from higher plants, insects, fungi and bacteria (Giardina et al., 2010). Fungal laccases, especially from wood decaying fungi, have been extensively characterized in comparison to others, where they are involved in delignification processes (Agrawal et al., 2018). Fungal laccases also play significant role in other physiological processes such as melanogenesis, stress management, defense mechanism and virulence (Zhu and Williamson, 2004).

3.1. Melanogenesis

Melanins are insoluble pigments formed by the oxidative polymerization of phenolic compounds catalyzed by phenoloxidase (laccases, catacholases or tyrosinases) and/or polyketide synthase. Melanins synthesized from exogenous diphenolic substrates such as 3, 4 dihydroxyphenylalanine (L- DOPA) are referred to as eumelanins and those synthesized from endogenous sources like acetate or malonate are known as dihydroxynaphthalene (DHN) melanin or allomelanin. The synthesis of both L-DOPA and DHN melanins in pathogenic fungi requires the activity of laccases (Eisenman et al., 2007; Frases et al., 2007). They form an important virulence factor in diverse pathogenic fungi to protect the cell from adverse environmental stressors, toxic reactive oxygen and nitrogen radicals, phagocytic killing by macrophages, host immune system and antifungal compounds (Nosanchuk and Casadevall, 2003; Coman et al., 2013).

Cryptococcus neoformans which is the casual agent of meningoencephalitis in HIV and other immunocompromised patients is the model organism in which effect of laccases on virulence against mammals has been extensively studied. Copper dependent and cell wall

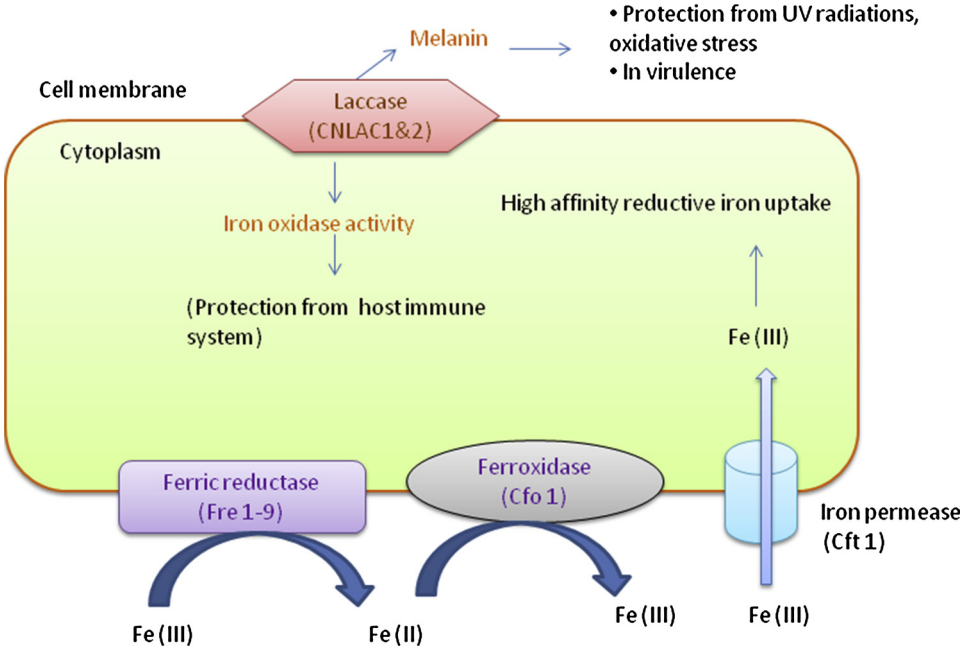

Fig. 2. Schematic representation of MCOs playing role in the virulence of *C. neoformans*. (Cfo 1: ferroxidase; Cft 1: iron permease; CNLAC1& 2: laccases). *C. neoformans* possesses high affinity reductive iron uptake system which involves the activity of two genes viz. cell surface ferric reductase (*fre1-8*) for the conversion of Fe (III) to Fe (II) ions and ferroxidase (*Cfo1*) which oxidizes Fe (II) to Fe (III) ions, further iron permease (Cft1) carries the oxidized iron ions to the cytoplasm. In addition, *C. neoformans* also possesses laccases (CNLAC1 and CNLAC2) as important virulence factors which oxidize mammalian substrates such as catecholamines into quinoles which further get polymerized into melanin pigment and confer protection against adverse conditions. It also posseses iron oxidase activity in macrophages for protection from toxic ions generated by host defense system.

Table 1
Ceruloplasmins and ferroxidases playing role in iron homeostasis mediated pathogenesis.

Multicopper oxidase	Source	Physical properties Temperature	pH	Kinetic constants	Functional properties	Role in pathogenesis		Reference
Ceruloplasmin	Humans	30 °C	5.0	Fe^{2+} K_m: 8.3 μM K_{cat}: 30.3 min^{-1} Cu^{1+} K_m: 36.8 μM K_{cat}: 22.5 min^{-1}	Ferroxidase activity Cuprous oxidase activity	Iron and copper metabolism	Mutant: Aceruloplasminemia: Defective iron and copper uptake. Iron deposition and injury in tissues Neurological deficits	Stoj and Kosman (2003)
Fet3p	*Saccharomyces cerevisiae*	37 °C	5.0	Fe^{2+} K_m: 2 μM p-phenylene diamine K_m: 900 μM o- phenylene diamine K_m: 4150 μM Epinephrine K_m: 5800 μM Cu^{1+} K_m: 38 μM K_{cat}: 79 min^{-1}	Ferroxidase activity p-phenylene diamine oxidase activity Amine oxidase acivity Cuprous oxidase activity	High affinity iron uptake Iron and copper homeostasis	Mutant: Defective iron uptake Copper redox imbalance	de Silva et al. (1997) Stoj et al. (2006)
CaFet3	*Candida albicans*	37 °C	6.0	Fe^{2+} K_m: 4.9 μM K_{cat}: 50.1 min^{-1}	Ferroxidase activity	High affinity iron uptake	Mutant: No growth in iron limited medium Defective iron uptake No change in virulence	Ziegler et al. (2011)
MCO	*Pseudomonas aeruginosa*	30 °C	5.0	–	Ferroxidase activity	Iron uptake	Defective Fe (II) acquisition No change in growth with Fe (III) solely present in minimal media No change in tolerance to copper ions	Huston et al. (2002)
Cfo1	*Cryptococcus neoformans*	–	–	–	Ferroxidase activity	High affinity reductive iron uptake Virulence	Mutant: Growth defect in iron depleted media Attenuated virulence Increased sensitivity to copper ions and antifungal drugs	Jung et al. (2009)

associated laccase enzyme (CNLAC1) mediates the synthesis of melanin pigment which contributes to be as one of the important virulence factor in this opportunistic pathogen. This pigment is being deposited in the outermost areas fungal cell wall by the oxidative polymerizations of exogenous substrates such as D and L enantiomers of 3, 4-dihydroxyphenylanine (DOPA), homogenestic acid and other *o*- and *p*-diphenolic substrates so that it can interact maximally with extracellular oxidants and resist toxic environment (Zhu et al., 2001; Eisenman et al., 2007). In mammalian hosts, neurotransmitters such as dopamine, epinephrine, norepinephrine are the substrates for laccase as they oxidize such catecholamines in brain into highly reactive *o*- quinine (DAQ) which is further converted to melanin via non-enzymatic mechanism. It is thought that melanin help in the survival of fungus in the host by impeding protection against innate and acquired immune system. Further knock out studies have justified the role of laccase in melanization and subsequent pathogenesis of *C. neoformans* as the mutant lacking the gene *CNLAC1* showed impaired melanization, defective dissemination and reduced virulence in murine model of cryptococcal infection (Zhu and Williamson, 2004). In addition, CNLAC1 possess an additional iron oxidase activity which protects the fungus from oxidative stress generated by reduced Fe (II) ions in mice alveolar macrophages (Zhu et al., 2004). Laccase mediated DOPA-melanin production also promotes virulence in other human pathogenic fungi such as *Candida albicans*, *Histoplasma capsulatum*, *Blastomyces dermatitisdis*, *Paracoccidioides brasiliensis* and *Coccidioides posadasii* (Taborda et al., 2008).

Laccases involved in the synthesis of DHN-melanin via polyketide synthase pathway are associated with conidial pigmentation and virulence. In human pathogenic fungi, *Aspergillus fumigates* and *A. nidulans*, laccases encoded by *Abr1/Abr2* and *yA* respectively oxidize and polymerize 1,8-DHN precursor to DHN melanin which generates pigmentation to infectious conidial structures and confer resistance to environmental and host stresses (Upadhyay et al., 2013). The transcriptional regulators viz. brlA, abaA, wetA are responsible for the process of conidiation in *Aspergillus*. Likewise, *Talaromyces marneffei*, a dimorphic opportunistic fungus which affects immuno-compromised individuals synthesize melanin pigments both *in vitro* and *in vivo*. A total of ten putative MCOs are being expressed in its genome, out of which *pbrB* gene encodes a laccase responsible for the synthesis of DHN melanin and green coloration of conidia during asexual development (Sapmak et al., 2015) (Table 2). Expression of laccase encoding melanin biosynthesis genes are upregulated only during conidiation and not in vegetative growth phase. Gene deletion mutants resulted in brown/yellow colored conidiophores which in turn affects fungal pathogenesis.

Melanization also play crucial role in virulence of phytopathogenic fungi such as *Colletotrichum orbiculare* and *Magnaporthe* sp. Oxidative activity of laccase (LAC2) of *C. orbiculare* is responsible for the polymerization of DHN melanin around the cell walls of appressorium. Melanin contributes rigidity and high turgor pressure to the appressoria for the penetration of host plant cuticle. Gene knock-out and host invasion studies of revealed that the lac2-mutants are non-pigmented, non-functional and non-pathogenic, suggesting the role of laccases in preinvasion of infection inside the plant host (Lin et al., 2012).

Certain bacterial strains have also opted for melanogenesis as a protective adaptation against adverse conditions. The most extensively studied laccase (CotA) from *Bacillus subtilis* is expressed in the endospore for the synthesis of the melanin-like pigment around spore coat that grant protection against UV light and H_2O_2 (Hullo et al., 2009). Cell membrane associated laccases are expressed by a number of nitrogen-fixing bacteria such as *Azotobacter chroococcum*, *Azospirillum lipoferum*, *Rhizobium* sp. during the encystement and melanogenic phase of growth when they encounter diverse environmental and nutritional stresses (Herter et al., 2011; Banerjee et al., 2014). Laccase is involved in the synthesis of melanin by other species of *Bacillus* as well such as *B. weihenstephanensis* (Drewnowska et al., 2015).

In a nutshell, the process of melanization provides protective advantage to microorganisms for survival inside the host and in other adverse environmental conditions. Also the ability of melanin to bind antimicrobial compounds and resist their mode of action, contributes a lot to the virulence of pathogens. Thus developing new antimicrobials by targeting the melanin biosynthesis pathways can be a novel pharmacological approach for combating pathogenesis which could influence the therapeutic interventions in future.

3.2. Stress adaptation

Laccase is one of the important multicopper oxidases involved in adaptation process in response to environmental adverse conditions. White rot fungi producing extracellular lignolytic enzymes, represent the largest fungal subgroup in which multiple fold increase in laccase activity was observed when subjected to different stress factors *in vitro*. Cho et al. (2009) studied the effect of diverse stress factors such as heavy metal ions, pro-oxidants and natural fungicides on laccase

Table 2
Laccases playing role in pigmentation mediated pathogenesis.

Multicopper oxidase	Source	Physical properties Temperature	pH	Role in pathogenesis		Reference
CNLAC1	*Cyptococcus neoformans*	30 °C	6.5	Melanogenesis Virulence	Mutant: Defective melanogenesis Impaired dissemination Attenuated virulence	Williamson et al. (1998) Zhu and Williamson (2004)
CotA	*Bacillus subtilis*	45 °C	7.0	Melanogenesis	Mutant: Loss of pigmentation Sensitivity to UV radiations and H_2O_2	Hullo et al. (2009)
Laccase	*Azotobacter chroococcum*	25 °C	5.0	Melanogenesis under nitrogen-fixing conditions	–	Herter et al. (2011)
pbrB	*Talaromyces marneffei*	37 °C	5.0	Pigmentation Virulence	Mutant: Delayed conidiation Sensitivity to H_2O_2, SDS, and antifungal agents Increased phagocytic killing by immune cells Attenuated virulence	Williamson (2016)
Abr 1 Abr 2	*Aspergillus fumigatus*	30 °C	7.0	Conidial pigmentation Virulence	Mutant: Defective melanin biosynthesis Attenuated virulence	Upadhyay et al. (2013)
LAC2	*Colletotrichum orbiculare* *Magnaporthe* spp.	–	–	Appressorial melanization Virulence	Mutant: Impaired melanization and pathogenicity	Lin et al. (2012)

activity of *Trametes versicolor* and *Abortiporus biennis* and found enhanced activity for the cellular adaptation and survival. Similarly biosynthesis of laccase in phytopathogenic fungus *Sclerotinia sclerotiorum* increased on exposure to chemical stress such as *Chelidonium majus* extract (antifungal compound) and choline derivatives of yeast extract suggesting the role of laccases in pathogenesis (Coman et al., 2013).

There are a number of reports where laccase has been considered as a part of adaptive response of fungal defense system against oxidative stress (Crowe and Olsson, 2001; Jaszek et al., 2006a, b; Cañero and Roncero, 2008; Tarhan and Tongul, 2017). Also, heterologous expression of laccase gene in yeast models could significantly increase their survival rate in the presence of oxidative stress mediated by H_2O_2 (Kim et al., 2006; Yang et al., 2012). The mechanism behind increased laccase activity and resistance to oxidative stress was studied by Yang et al. (2012) by expressing the laccase gene from white rot fungus *Trametes* sp. 5930 in yeast *Pichia pastoris*. Two yeast transformants were generated, the one containing recombinant plasmid (pPIC3.5K-lac5930-1) and the other lacking laccase gene (pPIC3.5K-GS115). They demonstrated that the stress generated by exogenous H_2O_2 lead to a higher degree of oxidative damage and accumulation of H_2O_2 in pPIC3.5K-GS115 as compared to pPIC3.5K-lac5930-1. It was also observed the laccase expression protects the yeast from oxidative damage by stimulating glutathione-based antioxidative system which scavenges H_2O_2 and other superoxide radicals generated. A notable increase in the expression of transcriptional regulator PpYAP1 was observed on laccase induction which in turn triggers the release of anti-oxidative enzymes viz. glutathione peroxidase, peroxisome glutathione peroxidase, glutathione reductases, γ-glutamylcysteine synthetase and confers protection against oxidative stress.

Evidence of laccases in brown rot fungi has been rare as they were considered to be deficient in laccase production (Machuca and Ferraz, 2001). But studies from genome sequencing confirms the presence of laccases in this group of wood rotting fungi too (Riley et al., 2014). Brown rot fungus *Postia placenta* MAD-R-698 expresses two isoforms of laccase (Pplcc1 and Pplcc2) which differ in their catalytic abilities. Pplcc1 is involved in wood decay mechanism whereas Pplcc2 is important for adaptation under stress conditions as an increase in the transcription of Pplcc2 was observed when subjected to chemical stress conditions (ethanol, ferulic acid and 2,6-dimethylbenzoic acid) (An et al., 2015) (Table 3). Thus all these findings strongly signify the competence of laccases in providing protection to the microbial cellular machinery against exogenous stress conditions.

3.3. Defense mechanism

Interaction of fungi with antagonistic microbes often results in hyphal modification in the zone of conflict and/or secretion of extracellular metabolites or oxidizing enzymes (Wei et al., 2010). Increase in laccase activity as a defense response was reported in intra-specific and inter-specific interactions of different white-rot fungi (Baldrian, 2004). During the antagonistic interspecific interactions between cultures of *Pleurotus ostreatus* and *Trichoderma longibrachiatum*, an increase in the transcription of laccase gene and subsequent enhanced activity was observed in *P. ostreatus* which limits the growth of *T. longibrachiatum* by the formation of laccase catalyzed zone of oxidized compounds (Velazquez-Cedeno et al., 2007). Inducing effect of *Trichoderma* on extracellular laccases was widely studied as a defense response in other basidiomycete fungi also such as *Agaricus bisporus, Lentinula edodes*, *Serpula lacrymans* and *Trametes versicolor*. (Bertrand et al., 2013). Similarly laccase (lcc2) from *Agaricus bisporus* was induced for the detoxification of lethal extract secreted by antagonistic fungus *Trichoderma aggressivum* and to confer protection against green mold disease (Sjaarda et al., 2015). In another competitive interaction of *T. viridae* with *Bacillus* sp. and *Aspergillus ochraceus*, high fold increase in laccase secretion was observed signifying the importance of laccase in combating antagonistic organisms and in fungal defense system (Lakshmanan and Sadasivan, 2016).

Not only laccase, but its catalyzed compounds also possess antimicrobial activities. Cinnabarinic acid, a laccase-catalyzed product of 3-hydroxyanthranilic acid from *Pycnoporus cinnabarinus*, acts as antibacterial agent by inhibiting the growth of *Streptococcus* (MIC 30–50 μg/ml) and *Klebsiella pneumoniae* (MIC 1.4 mg/ml) (Eggert et al., 1998). Similarly, iodination of phenolic compound vanillin with laccase resulted in the formation of products such as iodovanillin and iodoethylvanillin which have growth inhibitory effects against various wood decaying fungal species (Ihssen et al., 2014). Thus fungal laccases have been major players in defense response against stressful biotic conditions.

4. Multicopper oxidase (Pseudo-laccases): role in copper tolerance and virulence

Copper ions are essential for catalyzing various cellular and metabolic redox reactions and are important constituents of microbial enzymes such as cytochrome c oxidase, superoxide dismutase, amine oxidase and multicopper oxidase. However if in excess, they cause toxicity by generating super reactive hydroxyl radicals thereby damaging the macromolecules present in its vicinity (Fenton and Haber Weiss reactions) (Kehrer, 2000; Pham et al., 2013). At the same time Cu (I) can also disrupt the activity of certain metallo-proteins by displacing Fe-S clusters, thereby releasing free iron for enhanced oxidative toxicity. However, the toxic properties of copper have been used as a defense mechanism by the innate immune system to restrict the growth of microorganisms and combat infections (Djoko et al., 2015). Further, the expression of copper-carrier protein (ATP7A) is upregulated in response to pathogen for translocating sufficient amount of bactericidal copper in macrophages and/or to the site of infection (White et al., 2009). In response, pathogens have to rely on a series of transcriptional regulators which can activate copper tolerance genes for the survival and pathogenesis inside the host (Chaturvedi and Henderson, 2014). This section of the review discusses only the role of multicopper oxidases in attributing copper resistance and virulence to pathogenic bacteria. MCOs whose oxidation activity is dependent on Cu^{2+} are designated as pseudo-laccases. Such MCOs are induced and expressed in response to copper excess and are known as copper tolerance proteins. They have high cuprous oxidase activity and play significant role in copper homeostasis.

In *E. coli*, multicopper oxidase-CueO is a periplasmic enzyme which protects the cell from copper toxicity by working in conjugation with P-type ATPase-CopA. CopA is a metal ion-translocating ATPase that confers protection from cytoplasmic copper toxicity by delivering toxic Cu (I) to the periplasmic space and utilizing energy from ATP hydrolysis. Toxic Cu (I) ions are then oxidized to less toxic Cu (II) ions by the action of MCO-CueO (Fig. 3). The expression of these two genes is controlled by a MerR-like metallo-regulator (CueR) which is activated upon copper exposure only under aerobic conditions (Grass and Rensing, 2001; Singh et al., 2004). A unique feature of CueO is the requirement of fifth copper atom at its T1 site and the presence of an extra 14 methionines rich helix in domain (III) (Cortes et al., 2015). This forms an additional binding site for the copper atoms and exaggerates its role in copper resistance (Singh et al., 2011). Such methionine-rich copper binding region encoding for copper resistance is also present in plasmid encoded MCO-PcoA in *E. coli* (Djoko et al., 2008) MCO-CueO is also known to oxidize other substrates viz. ferrous ions, 2,6-dimethoxyphenol and siderophores. Under conditions of copper stress, enterobactin (indigenous siderophore) acts as Cu (II) reductant i.e. reduces less toxic Cu (II) ions to more toxic Cu (I) ions which in turn lead to cell damage by generating reactive oxygen species. (Grass et al., 2004). Oxidation of enterobactin and its catecholate precursors by CueO is an alternative mechanism for granting protection against copper toxicity. Also, oxidation of siderophores releases bound iron in the periplasm and helps in balanced transportation of divalent

Table 3
Laccases playing role in stress management and adaptation.

Stress factor (Concentration)	Source	Pathogenic adaptation	Reference
Oxidative stress			
Paraquat	*Trametes versicolor*	Increased expression of laccase, catalase, formaldehyde	Jaszek et al. (2006)
25 µM	*Abortiporous biennis*	Decrease in glutathione expression	
20 µM			
Hydrogen peroxide	*Coprinellus congregates*	Increased expression of laccase	Kim et al. (2006)
1.5M		Increased survival rate	
Hydrogen peroxide	Laccase from white rot fungus expressed in *Pichia pastoris*	Increased expression of laccase	Yang et al. (2012)
100 mM		Scavenge intracellular hydrogen peroxide	
		Protection from lipid oxidation damage	
Menadione	*Phanerochaete chrysosporium*	Increased expression of lignolytic enzymes viz. lignin peroxidase, manganese peroxidase and laccase.	Tarhan and Tongul (2017)
0.75 mM			
Heavy metal stress			
Cadmium	*Cerena unicolor* and *T. versicolor*	Increased activity of laccase, and formaldehyde	Jarosz-Wilkolazka et al. (1998)
25 mg/l			
Cadmium	*A. biennis*	Increased extracellular levels of laccase and intracellular levels of thiol compounds	Jarosz-Wilkołazka et al. (2006)
200 µM	*C. unicolor*		
50 µM			
Cadmium	*T. versicolor* and *Funalia trogii*	Increased expression of laccase	Mutlu et al. (2014)
100ppm			
Osmotic stress			
Ammonium sulfate	*Postia placenta* MAD-R-698	Increased expression of laccase	An et al. (2015)
0.2 M			
Sodium chloride			
0.2 M			
Mannitol			
0.6 M			
Biotic stress			
Trichoderma longibrachiatum	Antagonistic interactions between *Pleurotus ostreatus* and *Trichoderma longibrachiatum*	Increase in laccase activity	Velazquez-Cedeno et al. (2007)
		Inhibition of antagonistic growth	
Trichoderma aggressivum	Antagonistic interactions between *Agaricus bisporus* and *Trichoderma aggressivum*	Toxin metabolism by laccase	Sjaarda et al. (2015)
Bacillus sp	Antagonistic interaction of	Increase in laccase activity	Divya and Sadasivan (2016)
Aspergillus	*T. viridae* with *Bacillus* sp and *Aspergillus ochraceus*		
och raceus			
Chemical stress			
Cinnabarinic acid	*Pycnoporus cinnabarinus* antagonistic to *Streptococcus* (MIC 0.03-0.05 mg/ml) and *Klebsiella pneumonia* (MIC 1.4 mg/ml)	Transformation of 3- hydroxyanthranilic acid to cinnabarinic acid by laccase	Eggert et al. (1998)
		Antibacterial agent	
Chelidonium majus extract	*Sclerotinia sclerotiorum*	Upregulation of laccase activity	Coman et al. (2013)
Ethanol	*Postia placenta* MAD-R-698	Increased expression of laccase isoform Pplcc2	An et al. (2015)
0.20%			
Ferulic acid			
200 µM			
2,6-dimethylbenzoic acid			
200 µM			
Other stress factors			
Temperature	*Abortiporous biennis* (55 °C)	Increase in laccase activity	Jarosz-Wilkołazka et al. (2006)
	C. unicolor (45 °C)		
	T versicolor (45 °C)		
NSAIDs	*Yersinia enterocolytica*	Biotransformation of NSAIDs by laccase	Singh et al. (2016)
		Virulence	

cations via *feo* system (Kim et al., 2001). Thus MCO-CueO in *E. coli* forms an important component of copper homeostasis system and protects periplasmic enzymes from copper toxicity.

Likewise, copper-sensing transcriptional regulator CueR controls the expression of P-type ATPases-CopA and MCO-CueO in *Salmonella enterica* serovar *typhimurium*. CueO is responsible not only for conferring resistance to toxic copper ions but is also required for virulence in mouse model of systemic infection (Achard et al., 2010). It has been evident from the mutational and animal model studies that loss of CueO in a mutant strain resulted in impaired colonization in liver and spleen, though there was no difference in pathogen loads recovered from payer's patches, mesenteric lymph nodes and macrophages suggesting the role of CueO in attenuating virulence once the pathogen has disseminated inside the host.

Genes structurally and functionally homologous to *cueO* and *copA* of *E. coli* are also present in human food borne pathogen *Campylobacter jejuni* (*Cj1516* and *Cj1161* respectively) suggesting their role in copper homeostasis (Hall et al., 2008) (Fig. S2). MCO-Cj1516 in *C. jejuni* is a periplasmic enzyme as it possesses a 'twin-arginine' N-terminal signal sequence for its translocation and exihibits cuprous oxidase, *p*-phenylenediamine oxidase, ferroxidase and siderophore oxidation activities. The primarily function of MCO in *C. jejuni* is resistance to toxic copper ions which helps it to survive inside the host for pathogenesis. Similarly upregulation of putative copper resistance genes that contribute to copper tolerance was also observed in pathogens such as *Staphylococcus aureus* (Sitthisak et al., 2005), *Brucella melitensis* 16 M (Wu et al., 2015),

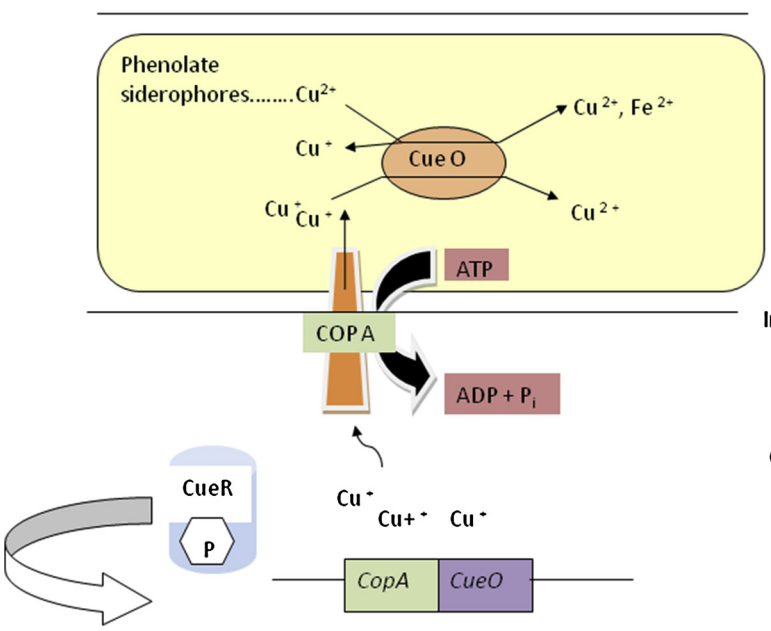

Fig. 3. Schematic representation of copper homeostasis in *E. coli*. (CueR: copper responsive metalloregulatory protein; CopA: copper exporting P-type ATPase; CueO: multicopper oxidase). CueR, a MerR family metaloregulator is expressed in response to copper ions entering the cell and initiates the transcription of *CopA* and *CueO* genes. Toxic Cu^{+} ions are transferred from cytoplasm to the periplasmic space with the help of ATP driven CopA pump and are oxidized into less toxic Cu^{2+} ions by multicopper oxidase CueO. Under copper stress, phenolate siderophore such as enterobactin exert toxicity in the cell by the reduction of Cu^{2+} to Cu^{+} ions. Oxidation of siderophores by CueO prevents from such enterobactin-Cu toxicity and also releases chelated iron thereby balancing the trafficking of divalent ions.

Acinetobacter baumannii (Williams et al., 2016) and *Desulfovibrio* sp. A2 (Mancini et al., 2017) (Table 4).

Copper defense is crucial for the virulence of intracellular pathogens such as *Mycobacterium tuberculosis* which can survive inside the toxic environment of macrophages. Thus this bacterium expresses a membrane associated MCO encoded by gene *Rv0846c* and annotated as MmcO which oxidizes toxic Cu ions and warrants its survival strategy inside the host (Rowland and Niederweis, 2013). Interestingly, MCOs are not expressed in the genomes of nonpathogenic *Mycobacterium*, suggesting they might have role in virulence. But loss of MmcO in mutant strain resulted in increased sensitivity to copper ions, but does not attenuate virulence of the pathogen. This can be implicated by the fact that though MCOs are involved in conferring tolerance to copper ions, but pathogens employ alternative mechanisms which can interplay and compensate the activity of mutated gene.

As such, loss of P-type ATPases which are responsible for cytoplasmic copper detoxification has been attributed to their role in copper hypersensitivity and attenuated virulence in many bacterial pathogens such as *Streptococcus pneumoniae*, *Listeria monocytogenes*, *M. tuberculosis*, *P. aeruginosa* and *S. enterica* pathovar *typhimurium* (Ladomersky and Petris, 2015). On the other hand, loss of MCO in *E. coli*, *C. jejuni*, *M. tuberculosis* and *B. melitensis* does not have impact on virulence (Hodgkinson and Petris, 2012; Wu et al., 2015). Thus in order to understand the basis of difference in role of copper tolerance genes in copper resistance and/or virulence, it is imperative to carry out the mutant studies by deleting the complete regulon responsible for copper homeostasis.

Copper resistance genes are also widespread in the genome of phytopathogens as they are being exposed to excessive amount of copper-containing bactericides in agricultural fields. In *Pseudomonas syringae* pathovar *tomato*, Cop proteins encoded by gene cluster *copABCD* sequester and accumulate periplasmic and outer membrane associated copper as a resistance mechanism against toxic copper ions (Huffman et al., 2002). Similarly, *copLAB* is the copper resistance gene present in *Xanthomonas campestris* pv. *campestris*. Deletion mutant studies demonstrated the role of CopA as a MCO and major contributor of copper resistance. Loss of *copA* not only rendered copper sensitivity but also attenuated virulence of this pathogen (Hsiao et al., 2011). In conclusion, MCOs encoding copper resistance genes have emerging role in copper tolerance and are attributed as important determinants of host-pathogen interactions.

5. Copper nitrite reductases: key role in nitrite respiration/ denitrification

Copper-containing nitrite reductases (CuNiRs) are the 2-domain class of multicopper oxidases which carry out single electron transfer for the reduction of nitrite to nitric oxide. This is an important, irreversible and committed step in the process of denitrification. (Nojiri et al., 2007). Cu-NiRs are periplasmic blue proteins encoded by *nirK* and widely distributed in denitrifying fungi such as *Fusarium oxysporum*, *Cylindrocarpon tonkinense* and *Aspergillus oryzae* (Kobayashi and Shoun, 1995; Nakanishi et al., 2010) and bacteria such as *Pseudomonas aureofaciens*, *Bacillus halodenitrificans*, *Bordetella*, *Alcaligenes xylosoxidans*, *Rhodobacter sphaeroides*, *Hyphomicrobium* sp, *Neisseria gonorrhoeae*, *Achromobacter cycloclastes* and *Geobacillus* sp. (Adman et al., 1995; Zumft, 1997). They play important role in anaerobic respiration of microorganisms i.e. help in the generation of energy via oxidative phosphorylation process being carried out using electron acceptor other than oxygen. In pathogens such as N*eisseria* sp., CuNir encoded by *aniA* gets induced under microaerophilic and/or anaerobic conditions, initiates an electrochemical potential in the presence of nitrite and helps in the growth and survival of pathogen inside the host (Mellies et al., 1997). In *Brucella melitensis*, NarA, a transcriptional regulator for the expression of genes involved in denitrification pathway viz. copper nitrite reductase (*nirK*), nitric oxide reductase (*norB*) and nitrous oxide reductase (*nosZ*), is essential for the virulence of this pathogen.

Presence of CuNiRs has also been investigated in many ammonia oxidizing bacteria such as *Nitrosomonas europaea* (NeNIR). These are categorized as green CuNirs as these carry out the process of denitrification under aerobic conditions. Such nitrite reductases have unique structure, different from typical CuNiRs but generate energy by converting nitrite to nitrous oxide at the same rate (Casciotti and Ward, 2001; Lawton et al., 2013). Thus such CuNiRs are oxygen tolerant and provide protection to the cell against the toxic effect of nitrite (Beaumont et al., 2002; Lawton et al., 2013). As such, structural insights and enzymatic mechanistics of CuNirs have been investigated thoroughly in the previous years (Li et al., 2015; Horrell et al., 2017), but their pathological role in microbes is not clear.

6. Conclusive remarks

Metals are extremely important for both the cellular and infectious

Table 4
Pseudo-laccases playing role in copper homeostasis mediated pathogenesis.

Multicopper oxidase	Source	Physical properties Temperature	pH	Cu^{2+}	Kinetic constants	Functional properties	Role in pathogenesis		Reference
YacK	*E. coli*	40 °C	5.0	1 mM	ABTS K_m: 2500 μM p-PD K_m: 3200 μM Fe^{2+} K_m: 70 μ M Enterobactin K_m: 40 μM 2,3-DHB K_m: 290 μM	Phenol oxidase activity Ferroxidase activity Oxidation of phenolate siderophores	Copper homeostasis	Mutant: Copper sensitivity Impaired uptake of divalent ions	Kim et al. (2001)
CueO	*E. coli*	– 25 °C	5.0 6.5	1 mM 0.5 mM	Cu^{1+} K_m:169 μM K_{cat}: 651 min^{-1} Fe^{2+} K_m: 129 μM K_{cat}: 215 min^{-1} Mn^{2+} K_m:17,330 μM K_{cat}: 0.034 min^{-1} Enterobactin K_m: 1.5 μM	Cuprous oxidase activity Phenol oxidase activity Ferroxidase activity Manganese oxidase activity Oxidation of enterobactin	Copper homeostasis	Mutant: Growth defects and copper sensitivity	Singh et al. (2004) Grass et al. (2004)
CopA	*Pseudomonas syringae*	–	–	0.1-0.5 mM	–	Methionine rich helix	Copper resistance		Cha and Cooksey (1993)
CutO	*Rhodobacter capsulatus*	25 °C	6.7	1 mM	DMP K_m: 1020 μM 2,3-DHB K_m: 150 μM Fe^{2+} K_{cat}: 17 min^{-1}	2,6-dimethoxyphenol oxidase activity Ferroxidase activity	Copper tolerance in both aerobic and anaerobic conditions	Mutant: Growth inhibition and sensitivity in the presence of copper under both aerobic and anaerobic conditions	Wiethaus et al. (2006)
pcoA	*E. coli*	25 °C	7.0	–	–	Methionine rich helix Phenol oxidase activity Cuprous oxidase activity	Copper resistance		Djoko et al. (2008)
Cj1516	*Campylobacter jejuni*	37 °C	5.7	1 mM	Cu^{1+} K_m: 180 μM Fe^{2+} K_m: 190 μM *p*-PD K_m: 270 μM 3,4-DHB K_m: 0160 μM	Cuprous oxidase activity Phenol oxidase activity Ferroxidase activity Siderophore oxidase activity	Copper homeostasis	Mutant: Copper sensitivity Unimpaired iron uptake	Hall et al. (2008)

(*continued on next page*)

Table 4 (*continued*)

Multicopper oxidase	Source	Physical properties Temperature	pH	Cu^{2+}	Kinetic constants	Functional properties	Role in pathogenesis		Reference
CueO	*Salmonella enterica* serovar *typhimurium*	37 °C	5.0	1 mM	Fe^{2+}	Cuprous oxidase activity	Copper tolerance	Mutant:	Achard et al. (2010)
					K_m: 52.6 μM k_{cat}: 16.11 min^{-1}	Ferroxidase activity	Virulence	Sensitivity to copper No change in bacterial colonization of lymph nodes, paeyer's patches and macrophages	
					Cu^{1+}			No sensitivity against oxidizing agents viz. H2O2, superoxide ions	
					K_m: 34.4 μM k_{cat}: 9.3 min^{-1}			Partially attenuated virulence	
CueO	*Desulfovibrio* DA2	30 °C	5.0	0.05 mM	Cu^{1+}				
					k_{cat}: 7.7 min^{-1} Fe^{2+}				
					k_{cat}: 182 min^{-1} DHB				
					k_{cat}: 2.3 min^{-1} ABTS				
					k_{cat}: 35min^{-1}				
CopA	*Xanthomonas campestris*	37 °C	5.0	0.1 mM	–	Multicopper oxidase activity	Copper resistance Virulence in the host cabbage.	Mutant: Decreased tolerance to copper Attenuated virulence	Hsiao et al. (2011)
MmCO	*Mycobacterium tuberculosis*	37 °C	5.5	0.25 mM	–	Cuprous oxidase activity	Copper resistance	Mutant	Rowland and Niederweis (2013)
						Ferroxidase activity		Copper susceptibility	
BmcO	*Brucella melitensis* 16M	37 °C	5.5	0.25 mM	–	Cuprous oxidase active	Copper tolerance	Mutant:	Wu et al. (2015)
						p-phenylenediamine oxidase activity		Susceptibility to copper	
						Ferroxidase activity		No role in intracellular growth	

processes. Metal homeostasis is a complex process and exerts selective pressure on pathogenic microorganisms for their survival inside the host. Recently, advances have been made in the field of molecular biology to study the interaction of metal ions at host-pathogen interface. Expression of metal sensing transcriptional regulators is upregulated for the maintenance of optimal concentrations of metal ions against the lethal consequences of metal overload and intoxication. Also, there is an emerging link between MCOs and their involvement in metal homeostasis systems. Thus the ability of MCOs to oxidize a broad range of substrates with high specificity towards metal ions makes them suitable for diverse protective roles in microorganisms. As such, they have been categorized as metalloxidases and impart tolerance to toxic metal ions and stressful environment.

MCOs are the new drug targets for the development and designing of novel antimicrobials. Genes encoding MCOs and their transcriptional factors interact with each other in a regulatory network for the functional activity of protein. System biology approach helps researchers to understand the relationship between gene dynamics and their regulatory network systems. This molecular interaction could be explored for the discovery of new drug targets. Another approach in the process of drug designing is to target the enzyme-substrate interaction. Homology modeling and molecular docking techniques could be very useful for the screening of compounds that can inhibit or block the active sites of enzyme. As MCOs are important virulence factors in the pathogenesis of many microbes, inhibiting their activity will eventually affect the survival mechanism and pathogenic events of microbes inside the host. These insights will help open new research areas in pharmaceutical microbiology field for the development of novel therapeutics using MCOs as drug targets.

Acknowledgement

The authors are highly grateful to the financial support provided by the Indian council of medical research (ICMR), New Delhi.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.02.007.

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