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Molecular modeling and docking of novel laccase from multiple serotype of *Yersinia enterocolitica* suggests differential and multiple substrate binding

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ABSTRACT

Multi-copper oxidases (MCOs) are widely distributed in bacteria, where they are responsible for metal homeostasis, acquisition and oxidation. Using specific primers, *yacK* coding for MCO was amplified from different serotypes of *Yersinia enterocolitica* biovar 1A. Homology modeling of the protein followed by docking with five well-known substrates for different MCO's (viz., 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid [ABTS], syringaldazine, L-tyrosine, ammonium ferrous sulfate and guaiacol), lignin monomers (Coniferyl alcohol, p-coumaryl alcohol and sinapyl alcohol) and two inhibitors i.e., kojic acid and N-hydroxyglycine was done. The docking gave maximum GoldScore i.e., 91.93 and 72.64 with ammonium ferrous sulfate and ABTS, respectively. Similarly, docking with ICM gave −82.10 and −83.61 docking score, confirming the protein to be true laccase with ferroxidase activity. Further, validation with ammonium ferrous sulfate as substrate gave laccase activity of 0.36 Units/L/min. Guaiacol, L-tyrosine, and lignin monomers showed good binding affinity with protein models with GoldScores of 35.89, 41.82, 40.41, 41.12 and 43.10, respectively. The sequence study of all the cloned *Yack* genes showed serotype specific clade in dendrogram. There was distinct discrimination in the ligand binding affinity of *Y. enterocolitica* laccase, among strains of same clonal groups, suggesting it as a tool for phylogenetic studies.

1. Introduction

Multicopper oxidases (MCOs) are a family of copper containing enzymes that includes laccase (EC 1.10.3.2), ferroxidases (EC 1.16.3.1), ascorbate oxidase (EC 1.10.3.3), ceruloplasmin monoxygenases, dioxygenases and various manganese oxidases [1–4]. These enzymes contain one to six copper atoms per molecule and about 100 to >1000 amino acid residues in a single peptide chain [5]. Laccases are defined as oxidoreductases that catalyze the reduction of molecular oxygen to water with the oxidation of a wide variety of phenolic and non-phenolic compounds [6]. Laccases have been found to evolve diverse role in plants, fungi, bacteria and insects [7]. In higher plants, these enzymes participate in the synthesis of lignin [8] whereas, in fungi, they play a role in lignin degradation, pigment formation, detoxification and pathogenesis [9–12]. Moreover, laccases has also been reported to catalyze the initial steps in melanin biosynthesis from diphenols in *Cryptococcus neoformans* thus protecting it from oxidative and microbicidal activities of host cell defense [13–16]. In bacteria, laccases are reported to participate in melanin production, spore coat resistance, involvement in morphogenesis and detoxification of copper [17–21]. The multi-copper oxidases (MCOs) are widespread in the members of the family enterobacteriaceae and other pathogenic bacteria with major role in copper homeostasis system in *Campylobacter jejuni*, *Escherichia coli* periplasm, *Vibrio cholera* and *Salmonella enterica* Serovar *Typhimurium*. In *Pseudomonas aeruginosa*, the MCOs, ferroxidases, have central role in iron acquisition [22–26] whereas, in other pathogenic bacteria like, *Staphylococcus aureus*, *Shigella dysenteriae* and *Klebsiella* sp. functions of the MCOs are yet to be discovered [27–29].

Yersinia enterocolitica is a gram-negative bacteria belonging to the family enterobacteriaceae. It causes a wide range of intestinal diseases, including enterocolitis with an inflammatory diarrhea, acute terminal ileitis [30] and mesenteric lymphadenitis mimicking appendicitis [31]. *Y. enterocolitica* is extremely heterogeneous serologically and is classified into six biovars (1A, 1B, 2, 3, 4 and 5) and more than 50 serotypes [32]. These are grouped on the basis

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of pathogenicity into highly pathogenic (biovar 1B), moderately pathogenic (biovars 2–5) and the so called non-pathogenic (biovar 1A) biovars [33].

Earlier, bioinformatics tools were used to report the presence of type 2 and type 3-multi copper oxidase and compare the amino acid sequence alignments in *Y. enterocolitica* and *Yersinia pestis*, respectively [19,23]. The present work aims to study putative laccase protein from diverse strains of *Y. enterocolitica* to reveal their phylogeny with known laccases and affinity with the true laccase substrate. This is the first report of MCO gene to be sequenced, modeled, analyzed and docked with different substrates, inhibitors and lignin monomers, to find out differential and multiple binding in different domains among serotypes of *Y. enterocolitica*.

2. Materials and method

2.1. Bacterial strains

In the present study, a total of 10 strains were selected from four different serotypes of *Y. enterocolitica* biovar 1A. Details of these strains have been reported previously [34]. Reference strain *Y. enterocolitica* 8081 (biovar 1B, serotype O:8) was provided to Dr. Virdi by Mikael Skurnik (Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Finland).

2.2. Evolutionary study

The putative laccase gene was amplified from genomic DNA by PCR amplification. Oligonucleotide primers, forward primer 5′ AT*GGATCCGAATTC*ATGCCATGCATCGCCGTGATTTTAT 3′, and reverse primer 5′ AA*GCGGCCGCCTCGAG*CTAAGCACTGACAGTAAGCC 3′ were designed from putative laccase gene of *Y. enterocolitica* subsp. *palearctica* 105.5R(r) (Acc No. NC_015224.1). To sequence the whole gene, walker primer 5′ TGGATGTAATGZCGGCTG 3′ was also designed. The amplified products were purified and cloned in TA cloning vector pTZ57R/T (Fermentas, India) and transformed into *E. coli* BL21 competent cells. The clones were confirmed by PCR and plasmid digestion using *Eco*RI and *Xho*I. Positive clones were sequenced and the data were analyzed using the NCBI databases and submitted to GenBank. Protein sequences from selected *Yersinia* strains of each serotype were used for the study of evolutionary relationships. All sequences were aligned using CLUSTAL W and phylogenetic relationships were inferred using the MEGA analysis tool v 5.1.

2.3. Physico-chemical characterization

The physico-chemical characterization like theoretical isoelectric point (pI), molecular weight, positively and negatively charged residues, extinction coefficient, instability index, aliphatic index and Gram Average Hydropathy (GRAVY) were computed using the Expasy Protparam server at http://web.expasy.org/protparam/ [35].

2.4. Homology modeling and molecular docking

The three dimensional structure of the multi-copper oxidase from different strains of *Y. enterocolitica palearctica* was predicted by homology modeling approach using crystal structure of *E. coli* multicopper oxidase taken from the protein data bank (PDB ID: 1KV7) as template by Modeller 9.10. The quality of each modeled structure for multicopper oxidase was validated using ProSa and SAVES server.

Molecular docking was done using two softwares viz., CCDC GOLD (Genetic Optimization for Ligand Docking) and Molsoft ICM (Internal Coordinate Mechanics) software. The docking performed by GOLD software was carried out with hundred genetic algorithms (GA) run for each compound. In a single GA runs, 1,00,000 operations were performed on a population size of 100 individuals with a selection pressure of 1.1. The number of islands was set to 5 with a niche size of 2. The values for crossover, mutation and migration were set as 95, 95 and 10, respectively. Using ICM software, flexible ligand docking was carried out by optimizing a set of ligand internal coordinates in the space of grid potential maps calculated for the protein pocket using Monte Carlo simulations. The ICM scoring function is weighted according to the parameters like, internal force-field energy, entropy loss between bound and unbound states of the ligand, ligand—receptor hydrogen bond interactions, polar and non-polar solvation energy differences between bound and unbound states, electrostatic energy, hydrophobic energy, and hydrogen bond donor or acceptor desolvation. Generally a score below -32 is regarded as a good docking score. The lower the ICM score, the higher the chance the ligand is a binder.

Ten ligands were used for binding/docking with the receptor which included MCO substrates ABTS, syringaldazine, guaiacol, L-tyrosine, ammonium ferrous sulfate, inhibitors, kojic acid and N-hydroxyglycine and lignin monomers coniferyl alcohol, p-coumaryl alcohol and sinapyl alcohol. The *in silico* docking results were validated using ammonium iron(II) sulfate as a substrate for laccase assay [22]. Enzyme activity was calculated using an extinction coefficient for Fe(III) of 2.2 mM^{-1} cm^{-1} at 315 nm.

3. Results

3.1. Evolutionary properties of Yersinia enterocolitica putative laccases

Gene amplification with specific primers showed the band size of 1.6 Kb (Fig. 1). The analysis of complete sequences of gene showed 96% sequence homology with previously submitted multicopper oxidase gene from *Y. enterocolitica* subsps. *enterocolitica* 8081 and were submitted to GenBank under accession number KC113580–KC113589 (Table 1). Of all the strains studied, strain E13 (Serotype O:6-30-6-31) gave no PCR amplification for the gene probably due to missing ORF. The neighbor joining phylogenetic tree of multicopper oxidase from different pathogenic bacteria showed that the MCO's of serotype O:10-34 (strains E15 and 53) are very similar and share a common clade. Although, the MCO's of different strains of *Y. enterocolitica* do not show distinct differentiation on the basis of serotypes, but strains E7 and 43 of serotype O:6-30 are in the same clade as the strains 11 and 12 of serotype O:6-30-6-31 share same clade. Despite being from the different serotypes, the MCO's of strain E11 (O:6-30-6-31) and E5 (O:6-30) are in the same clade, which predicts them to be very similar and the MCO of strain 7 (O:6-30-6-31) was also similar to them. The MCO of strain E6 (O:5) and strain 8081 (O:8) are different from the others (Fig. 2a). The Bootstrap analysis (NJ, 2000 replicates) on the combined data set support all the branches with high value (70–100%) except two short internal branches (54% and 61%, respectively). The phylogenetic tree based on protein domains showed highly conserved region in the strains belonging to same serotypes, as well as clonal groups (Fig. 2b) and its Bootstrap analysis also supported all the branches with high value (72–99%) except for a short internal branch (54%).The sequence analysis using the TatP [36] and SignalP [37] web servers and comparison with previous bacterial MCO's shows the presence of TAT pathway region, which suggests that the protein is secreted into the periplasm due to the presence of a typical consensus conserved Tat signal motif (Fig. 3). The cleavage is predicted to remove 30 amino acids during export from the 5′-end of the signal peptide.

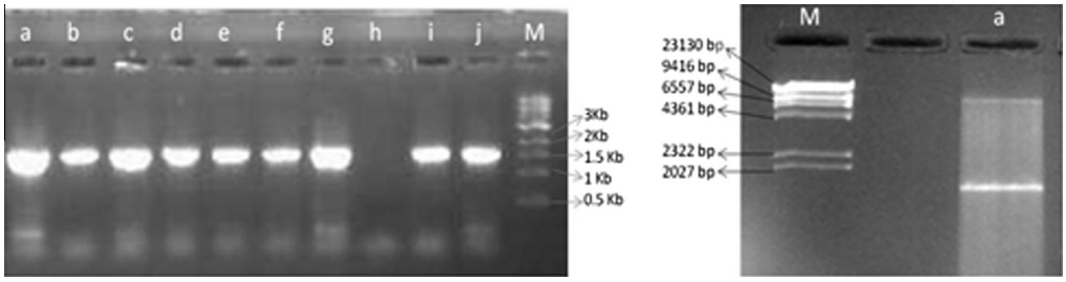

Fig. 1. (A) Agarose gel (1%) electrophoresis showing band of laccase sequence of: *Yersinia enterocolitica* subsp. *Palearctica* [a] strain 7 [b] strain 11 [c] strain 12 [d] strain E11 [e] strain E15 [f] strain 53 [g] strain 43 [h] strain E13 [i] strain E6 [j] strain 12 [M] 1 Kb Marker. (B) [a] TA cloning vector (pTZ57R/T) with laccase and [M] Lambda DNA Hind III digest marker.

Table 1
Details of the strains of *Yersinia enterocolitica* biotype 1A used in the present study.

S. No.	Organism	GenBank accession No. (multicopper oxidase)	Serotype	Country of origin	Source of origin	Instability index	Aliphatic index	GRAVY Score
1	*Yersinia enterocolitica* subsp. *Palearctica* strain 7	KC113580	0:6,30-6,31	India	Human stools	38.17	83.32	−0.120
2	*Yersinia enterocolitica* subsp. *Palearctica* strain E11	KC113583	0:6,30-6,31	France	Human	38.22	83.29	−0.123
3	*Yersinia enterocolitica* subsp. *Palearctica* strain 11	KC113581	0:6,30-6,31	India	Waste water	38.45	83.51	−0.114
4	*Yersinia enterocolitica* subsp. *Palearctica* strain 12	KC113582	0:6,30-6,31	India	Waste water	38.45	83.51	−0.114
5	*Yersinia enterocolitica* subsp. *Palearctica* strain 43	KC113588	0:6,30	India	Human stools	37.65	83.51	−0.118
6	*Yersinia enterocolitica* subsp. *Palearctica* strain E7	KC113587	0:6,30	France	Human	37.65	83.51	−0.117
7	*Yersinia enterocolitica* subsp. *Palearctica* strain E5	KC113586	0:6,30	NK	NK	38.17	83.32	−0.119
8	*Yersinia enterocolitica* subsp. *Palearctica* strain 53	KC113584	0:10-34	India	Wastewater	38.64	84.97	−0.095
9	*Yersinia enterocolitica* subsp. *Palearctica* strain E15	KC113585	0:10-34	France	Human	39.01	84.97	−0.097
10	*Yersinia enterocolitica* subsp. *Palearctica* strain E6	KC113586	0:5	NK	Patient	38.76	83.86	−0.110
11	*Yersinia enterocolitica* subsp. *Palearctica* strain 8081	YP_001005057.1	0:8	USA	Human	38.15	82.78	−0.137

3.2. Physico-chemical characterization

The physico-chemical analysis gave a theoretical isoelectric point (pI) value of 5.97 for the MCO of all the strains, except for the strains of serotype O:10-34 (strain E15 and 53), which had a pI value of 6.13, slightly higher than the others. The molecular weight of all the proteins was uniform. The positively and negatively charged residues were also alike for all the proteins, except for strains E15 and 53. The negative GRAVY score predicts the protein to be hydrophilic. All the stains have proteins with instability index less than 40 thereby indicating stability. The aliphatic index for all the strains was found to be in the range of 83.29–83.97, which suggests that the protein is thermostable (Table 1, Supplementary data Table 1).

3.3. Homology modeling and molecular docking

The homology modeling of the *Y. enterocolitica* MCO gene sequences obtained from previous experimental data showed best (66%) similarity with 1KV7, chain A crystal structure of CueO, a multicopper oxidase from *E. coli* involved in copper homeostasis with a resolution of 1.4 Å. The models generated were verified for quality check which showed a Ramachandran plot value of 80–85% residues in allowed region and a *Z*-score value of −1.64 to −3.91 (Supplementary data Table 2). The above data authenticates the structures appropriate for further use in molecular docking.

Molecular docking gave maximum Goldscore of 91.93 and 72.64, respectively, with substrates ammonium ferrous sulfate and ABTS. The binding affinity with guaiacol, L-tyrosine and lignin monomers were also good with a Goldscore of 35.89, 41.82, 40.41, 41.12 and 43.10, respectively. Docking with molsoft ICM software also gave similar results with maximum interaction of protein with substrates ammonium ferrous sulfate and ABTS having a docking score of −82.10 and −83.61, respectively. Substrates, syringaldazine, guaiacol, L-tyrosine, and lignin monomers, coniferyl alcohol, p-coumaryl alcohol and sinapyl alcohol, also gave good docking score of −78.24, −44.68, −55.51, −58.50, −51.86, −62.90, respectively. The interaction of the inhibitor kojic acid with the MCO was similar to the substrates guaiacol, L-tyrosine and lignin monomers, with a GoldScore of 38.40 and 26.65, respectively and an ICM docking score of −48.93 and −33.05, respectively (Supplementary data Table 4 and Fig. 1). There was no distinct discrimination on the binding affinity of the MCO's of *Y. enterocolitica* strains with the ligands on the basis of serotypes. But the strains behaved differentially on the basis of previously defined clonal groups (Fig. 4).

The assay results showed that MCO from strain 7, 12, E14 and 8081 of *Y. enterocolitica* had an enzyme activity of 0.079, 0.32, 0.32 and 0.36 Units/L/min, respectively, with the substrate ammonium ferrous sulfate. These results validate the *in silico* docking results.

4. Discussion

Present study was focused on the evolutionary analysis of *yack* gene, which codes for MCO in different serotypes of *Y. enterocolitica* biovar 1A and the interaction of substrates of MCO with the *in silico* translated protein. Earlier studies differentiated *Y. enterocolitica* biovar 1A into two clonal groups (clonal group A and clonal group B) on the basis of various genotyping techniques [34,38,39], chromosomal restriction analysis (ribotyping) and rRNA spacer length polymorphism [38]. Other tools like multilocus variable number tandem repeat (MLVA) analysis [39], multilocus enzyme electrophoresis (MLEF), multilocus restriction tying (MLRT) [38] and whole cell protein profiling [33] were used by Virdi and co-workers to strengthened the differentiation of strains of *Y. enterocolitica* biovar 1A into two clonal groups. The comparison of amino acid sequences of MCO and construction of phylogenetic tree revealed high similarity among strains of same serotypes, corroborating with the earlier results based on rep(REP/ERIC)-PCR genomic

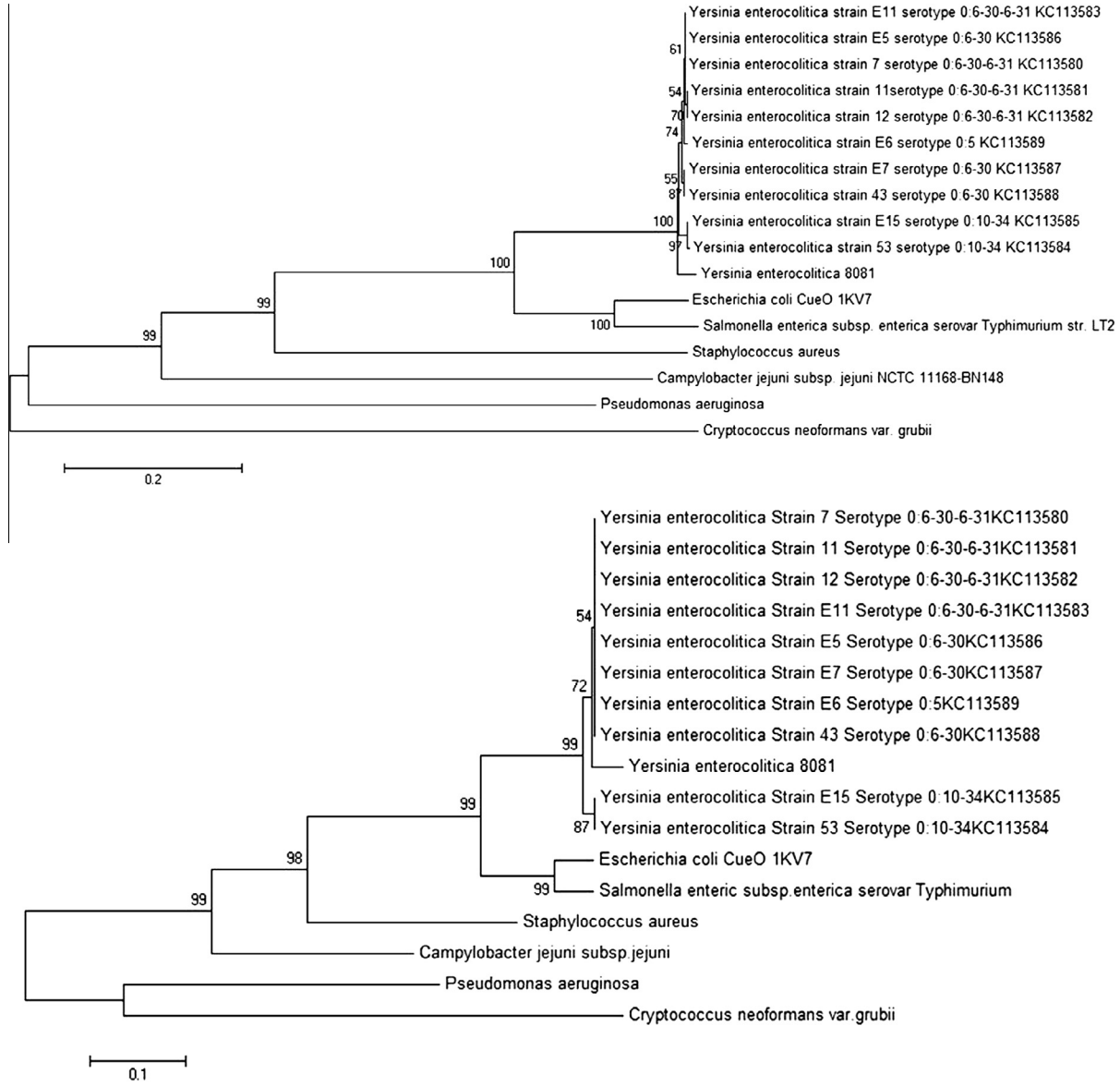

Fig. 2. Phylogenetic tree of (a) laccase (b) domains of laccase, from diverse strains of selected serotypes of *Y. enterocolitica* using Mega 5 software. *Y. enterocolitica* 8081 was used as reference strain.

fingerprinting. The serotypes belonging to clonal group A shared same clade, whereas clonal group B were in different clade. The *Yersinia* laccase was very similar to multicopper oxidases of other bacteria and yeast, that are either pathogens or opportunists e.g., basidiomycetes yeast *C. neoformans* and bacteria *S. enterica serovar Typhimurium* [15,25]. Further, the MCO substrates interaction with *in silico* generated protein model from various strains of *Y. enterocolitica* showed similar binding pattern of copper atoms coordinating to similar amino acids as in MCO of different bacteria, like *E. coli* and *C. jejuni*, which were also found to be consensus conserved as in fungal and previously reported bacterial laccases, indicating MCO from *Y. enterocolitica* to be a stable putative laccase.

Earlier studies have proved that type I copper atom is constructed from 1Cys2His and weakly coordinating 1Met or the non-coordinating 1Phe/1Leu [40]. The type I Cu of all the MCOs in this study showed coordination with 2His, 1Cys and 1Leu whereas, the pathogenic bacteria showed additional coordination of Type-I Cu with 1Met. The methionine rich region in bacterial multicopper oxidases have earlier been reported to have role in blocking the solvent access to the T1 site in the absence of excess copper [41], thus contributing ligands to an additional copper-binding site that must be occupied for full CueO activity [42]. Further characterization of copper centers in MCO protein of all the strains showed highly conserved amino acids in domain I, II and III, whereas, domain IV was found to be more conserved among strains of same serotypes. Moreover, the copper binding amino acids of clonal group B strains were more similar to the strain 8081 and other pathogenic bacteria. This high similarity indicates the possibility that strains of clonal group B can evolve or transform into potential pathogens.

Moreover, the docking pattern and GoldScore was found to be similar for all the strains. Differential evolution of active-sites region of protein and the individual domains of a particular protein, infers that active-site is more conserved to perform a specific or unique reaction and scaffold region is variable. This variable region was used as a taxonomic marker to study the phylogenetic position by *in silico* protein modeling. The GOLD average fitness score for ABTS of our studies with laccase protein are similar to their previous report by Kumar and co-workers [43], indicating

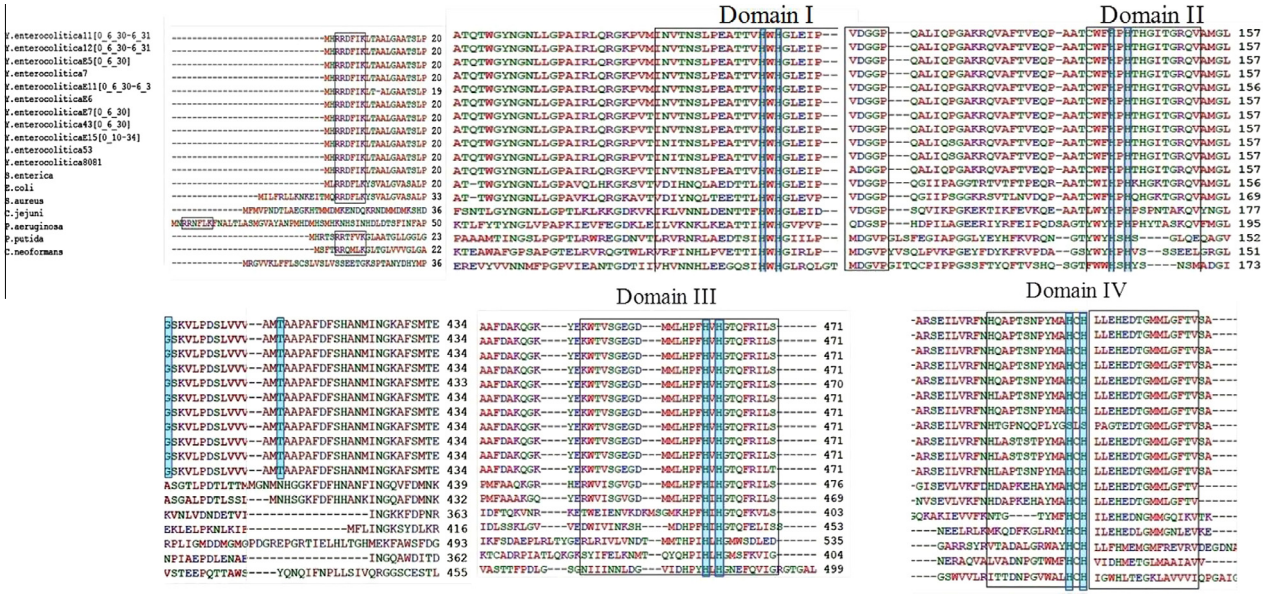

Fig. 3. Amino acid sequence alignments for four copper-binding domains of *Y. enterocolitica* strains and comparison with other selected bacterial MCO's. The twin arginine signal motif (TAT) are shown in shaded boxes. Alignments were performed using Clustal W. * shows glycosylated amino acids.

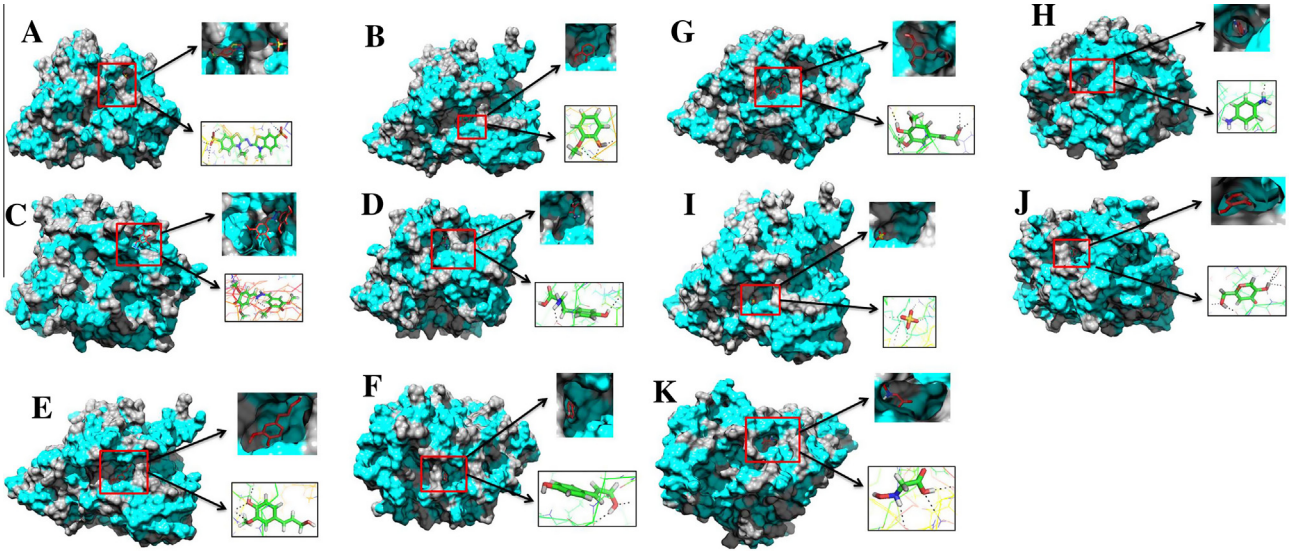

Fig. 4. Docking of different substrates and inhibitors with MCO's of different strains of *Y. enterocolitica* using GOLD software. The blue color shows the hydrobhobic residues and the grey color shows the hydrophilic residues of the MCO's. The interaction of the substrate/inhibitor and the close view of binding site pocket with substrate/inhibitor is shown in the inset. Docking of substrate and MCO with best GOLD score (A) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) with strain 43; (B) Guaiacol with strain 8081; C. Syringaldazine with strain E11; (D) L-Tyrosine with strain E6; (E) Coniferyl alcohol with strain 8081; (F) p-Coumaryl alcohol with strain 12; (G) Sinapyl alcohol with strain E7; (H) Para-phenylenediamine with strain E15; (I) Ferrous ammonium sulfate with strain 8081 and Inhibitors. (J) Kojic acid with strain 11 k. N-hydroxyglycine with strain E7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ABTS an appropriate substrate for laccase. Earlier, ammonium ferrous sulfate has been used as a substrate by several workers for ferroxidase assay [22,26]. Ferroxidase, plays significant role in iron uptake of prokaryotes and eukaryotes, catalyzing the oxidation of Fe(II) to Fe(III) followed by its transfer into the cell with the help of iron permeases [4,26]. The docking results were validated by using ammonium ferrous sulfate as a substrate for MCO from *Y. enterocolitica*. Our results for lignin monomers Coniferyl alcohol, p-coumaryl alcohol and sinapyl alcohol show good binding affinity with MCO protein model.

Earlier, Faure and coworkers [44] has reported that N-hydroxyglycine inhibits *Azospirillum lipoferum* laccase and kojic acid inhibits all the polyphenol oxidases. Also, ptilomycalin A, an antifungal spirocyclic guanidine alkaloid, inhibits the biosynthesis of laccase and thus melanogenesis of *C. neoformans* [45]. The interaction of the inhibitors i.e., kojic acid and N-hydroxyglycine with putative laccase was similar to the substrates studied. Thus, MCO i.e., laccase can be used as drug target in pathogenic microorganisms with MCO as a virulence factor. These inhibitors like tetrathiomolybdate, kojic acid and N-hydroxyglycine [45–47] may act by competing and blocking substrate binding site thus inhibiting the action of laccase. So, there is possibility that it can be used as a selective drug target for MCO inhibition and eventually pathogenesis. Further, more work need to be done to validate the role of laccase in drug targeting.

Laccase gene from *Y. enterocolitica* has shown serotype specific variations, but the protein shows variation among different clonal groups leading to changes in secondary structures and thus

differential ligand binding due to slight changes in amino acids among different strains. Further, docking experiments give the idea of potential substrate for MCO's from *Y. enterocolitica*. Moreover, docking with different substrates and inhibitors, especially with ABTS and ferrous ammonium sulfate, as a substrate showed good score value which suggests protein to be a true laccase, which have been earlier, reported as a probable factor for pathogenicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.003.

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