Properties of a cryptic lysyl oxidase from haloarchaeon *Haloterrigena turkmenica*

Nikolay B Pestov Corresp., [1] , **Daniel V Kalinovsky** [1] , **Tatyana D Larionova** [1] , **Alia Z Zakirova** [1] , **Nikolai N Modyanov** [2] , **Irina A Okkelman** [1] , **Tatyana V Korneenko** [1]

[1] GCLE, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia

[2] Department of Physiology and Pharmacology, University of Toledo, Toledo, OH, United States

Corresponding Author: Nikolay B Pestov
Email address: korn@mail.ibch.ru

B ackground: Lysyl oxidases (LOX) have been extensively studied in mammals, whereas properties and functions of recently found homologues in prokaryotic genomes remain enigmatic. **Methods:** LOX open reading frame was cloned from *Haloterrigena turkmenica* in an *E. coli* expression vector. Recombinant HTU-LOX proteins were purified using metal affinity chromatography under denaturing conditions followed by refolding. Amine oxidase activity has been measured fluorometrically as hydrogen peroxide release coupled with the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine in the presence of horseradish peroxidase. Rabbit polyclonal antibodies were obtained and used in western blotting. **Results:** Cultured *H. turkmenica* has no detectable amine oxidase activity. *H. turkmenica* lysyl oxidase (HTU-LOX) may be expressed in *E. coli* with a high protein yield. The full-length protein gives no catalytic activity. On the other hand, truncated His-tagged HTU-LOX lacking the N-terminal hydrophobic signal peptide purified under denaturing conditions can be successfully refolded into an active enzyme, and a larger N-terminal truncation further increases the amine oxidase activity. Refolding is optimal in the presence of Cu^{2+} at pH 6.2 and is not sensitive to salt. HTU-LOX is sensitive to LOX inhibitor β-aminopropionitrile. HTU-LOX deaminates usual substrates of mammalian LOX such as lysine-containing polypeptides and polymers. The major difference between HTU-LOX and mammalian LOX is a relaxed specificity of the former. HTU-LOX readily oxidizes various primary amines including such compounds as taurine and glycine, benzylamine being a poor substrate. Of note, HTU-LOX is also active towards several aminoglycoside antibiotics and polymyxin. Polyclonal antibodies against HTU-LOX detect a band with the molecular weight corresponding to a full-length product among *H. turkmenica* proteins. **Conclusion:** *H. turkmenica* contains a lysyl oxidase gene that may be heterologously expressed yielding an active recombinant enzyme with important biochemical features conserved between all known LOXes, for example, the sensitivity to β-aminopropionitrile. However,

the native function in the host appears to be cryptic. **Significance:** This is the first report on some properties of a lysyl oxidase from Archaea and an interesting example of evolution of enzymatic properties after horizontal transfer between distant taxa.

Properties of a cryptic lysyl oxidase from haloarchaeon *Haloterrigena turkmenica*

Nikolay B. Pestov[1], Daniel V. Kalinovsky[1], Tatyana D. Larionova[1], Alia Z. Zakirova[1], Nikolay N. Modyanov[2], Irina A. Okkelman[1], Tatyana V. Korneenko[1]

[1] Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia

[2] University of Toledo College of Medicine, Toledo, Ohio

* Short title: *Archaeal Lysyl Oxidase*

To whom correspondence should be addressed: Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Miklukho-Maklaya 16/10, Moscow, 117997, Russia, Tel: +7(495) 330-6556; Fax: +7(495) 330-6556; E-mail: korn@mail.ibch.ru

Keywords: amine oxidase, archaea, halophiles, horizontal gene transfer, lysyl oxidase

ABSTRACT

Background: Lysyl oxidases (LOX) have been extensively studied in mammals, whereas properties and functions of recently found homologues in prokaryotic genomes remain enigmatic. **Methods:** LOX open reading frame was cloned from *Haloterrigena turkmenica* in an *E. coli* expression vector. Recombinant HTU-LOX proteins were purified using metal affinity chromatography under denaturing conditions followed by refolding. Amine oxidase activity has been measured fluorometrically as hydrogen peroxide release coupled with the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine in the presence of horseradish peroxidase. Rabbit polyclonal antibodies were obtained and used in western blotting. **Results:** Cultured *H. turkmenica* has no detectable amine oxidase activity. *H. turkmenica* lysyl oxidase (HTU-LOX) may be expressed in *E. coli* with a high protein yield. The full-length protein gives no catalytic activity. On the other hand, truncated His-tagged HTU-LOX lacking the N-terminal hydrophobic signal peptide purified under denaturing conditions can be successfully refolded into an active enzyme, and a larger N-terminal truncation further increases the amine oxidase activity. Refolding is optimal in

the presence of Cu^{2+} at pH 6.2 and is not sensitive to salt. HTU-LOX is sensitive to LOX inhibitor β-aminopropionitrile. HTU-LOX deaminates usual substrates of mammalian LOX such as lysine-containing polypeptides and polymers. The major difference between HTU-LOX and mammalian LOX is a relaxed specificity of the former. HTU-LOX readily oxidizes various primary amines including such compounds as taurine and glycine, benzylamine being a poor substrate. Of note, HTU-LOX is also active towards several aminoglycoside antibiotics and polymyxin. Polyclonal antibodies against HTU-LOX detect a band with the molecular weight corresponding to a full-length product among *H. turkmenica* proteins. **Conclusion:** *H. turkmenica* contains a lysyl oxidase gene that may be heterologously expressed yielding an active recombinant enzyme with important biochemical features conserved between all known LOXes, for example, the sensitivity to β-aminopropionitrile. However, the native function in the host appears to be cryptic. **Significance:** This is the first report on some properties of a lysyl oxidase from Archaea and an interesting example of evolution of enzymatic properties after horizontal transfer between distant taxa.

INTRODUCTION

Lysyl oxidase is an amine oxidase that is well characterized in mammals. The human genome contains five lysyl oxidase isoforms (LOX and LOXL1-4), all of them possess the highly conserved C-terminal catalytic domain, the N-terminal signal peptide, and the accessory segments in between. LOX catalytic domain is unique among other mammalian amine oxidases because of its ability to oxidatively deaminate various amines including ε-amino groups of lysine residues in peptides and proteins. LOX activity initiates cross-link formation between certain proteins, including elastin, collagen, and fibronectin, and this process is important for maturation and remodeling of extracellular matrix (Grau-Bove et al, 2015).

Animal genomes sequenced to date, except for nematodes and ctenophores, contain from one to five LOX genes, whereas plants and most fungi are unknown to possess it. LOX genes are also absent from the vast majority of prokaryotic genomes. Therefore, the presence of true homologues of animal LOX in just several species of Eubacteria and Archaea is of significant interest; it reflects the unique history of this enzyme – LOX genes underwent multiple horizontal transfer (HGT), and are frequent in Actinomycetes (especially Streptomycetes), some

Deltaproteobacteria, occasionally – in other eubacteria, and very rarely – among Archaea. This aspect is exciting not only from the phylogenetic point of view, but also because of potential biotechnological applications, i.e. the fact that distantly related enzymes may have useful properties *(Noda-García et al, 2013).*

It is interesting to note that, in contrast to eukaryotic lysyl oxidases, several LOX homologues identified in prokaryotes exhibit a simple architecture often without a signal peptide (*Grau-Bove et al, 2015*). On the other hand, some prokaryotic LOXes are more complex. Specifically, LOX from *Sorangium cellulosum* possesses a unique Cys-rich C-terminal non-catalytic domain, which is presumably highly disulfide cross-linked.

The few lysyl oxidase homologues from Archaea that have been sequenced are clustered in two independent groups. This suggests that Thaumarchaeotes and Euryarchaeotes may had acquired LOX genes in two independent HGT events (*Grau-Bove et al, 2015*). This is just an example of widespread HGT in Archaea (*Papke et al, 2015). Haloterrigena turkmenica* was isolated from Turkmenistani sulfate saline soil by Zvyagintseva and Tarasov and described in 1987 as *Halococcus turkmenicus* (*Zvyagintseva and Tarasov, 1987*). In 1999 it was proposed to rename it to *Haloterrigena* (*Ventosa et al, 1999*). *H. turkmenica* belongs to the family Halobacteriaceae typus Euryarchaeota and is a fairly fast growing chemoorganotrophic extreme halophile that requires at least 2 M NaCl with optimal temperature around 45°C. The complete genome of this archaeon has been sequenced. It consists of 5,440 kbp (including plasmid 6) and it was annotated as encoding 5,287 proteins and 63 ncRNAs *(Saunders et al, 2010*).

Here, we attempted for the first time a study on the properties of lysyl oxidase from this archaeon.

EXPERIMENTAL PROCEDURES

Materials and strains. A fresh stock of *Haloterrigena turkmenica VKMB-1734* was purchased from the All-Russian Collection of Microorganisms (G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Pushchino, Moscow Region, Russia).

Cultivation of H. turkmenica. Various haloarchaeal media with NaCl around 200 g/l such as INMI medium-3, DSMZ-372 are suitable. Care should be taken to adjust pH since *H. turkmenica*

does not grow in acidic media. We found that a simpler medium (hereafter referred to as **IAO**) is a better choice: casamino acids – 5 g/l, yeast extract – 5 g/l, NaCl – 220 g/l, pH 7.6 - autoclaved and supplemented with $MgSO_4$ – 5 mM, $CuCl_2$ – 10 μM. Solid IAO medium may be used for growing single colonies, however, only with high quality agar (some batches inhibit growth). Also, *H. turkmenica* can be easily adapted to a defined medium, hereafter referred to as **MHTU** (modified HMM from (*Mosin and Ignatov, 2014*)) – modified from L-alanine – 0.4, L-arginine – 0.4, D-asparagine – 0.2, L-aspartic acid – 0.4, L-cysteine – 0.1, L-glutamic acid – 1.5, L-histidine – 0.7, L-isoleucine – 0.5, L-leucine – 0.8, D,L-lysine 2, D,L-methionine – 0.4, L-phenylalanine – 0.3, L-proline – 0.4, D,L-serine – 0.6, L-threonine – 1, L-tyrosine – 0.2, D,.L-tryptophan – 0.5, L-valine – 1, AMP – 0.1, NaCl – 220, $MgSO_4$-$7H_2O$ – 20, KCL – 2, NH_4Cl – 0.5, KNO_3 – 0.1, KH_2PO_4 – 0.1, K_2HPO_4 – 0.1, Na_3-citrate – 0.8, $MnSO_4$-$2H_2O$ – 0.0003, $CaCl_2$-$6H_2O$ – 0.1, $ZnSO_4$-$7H_2O$ – 0.00005, $FeSO_4$-$7H_2O$ – 0.00005, $CuCl_2$ – 10 μM, glycerol – 1, D-leucine-OH – 0.1, norleucine – 0.1, thymine – 0.1, uracil – 0.1, pH 7.5.

Gene cloning. The DNA used as a template for PCR was isolated from the cell culture using a ZR Fungal / Bacterial DNA MicroPrep kit (Zymo Research, USA) according to the manufacturer's instructions. For PCR with Taq-polymerase, 0.8 μM of "direct" and "reverse" primers, 10x *Taq* PCR buffer, 2.5μl 2 mM solution of deoxyribonucleotide triphosphates, 0.1 μl of Taq DNA polymerase, *H. turkmenica* genomic DNA as a template and water were mixed in 25μl of total reaction volume. For PCR with Phusion-polymerase in an equal volume of 25 μl, corresponding "direct" and "reverse" primers at a concentration of 0.8 μM, PCR buffer 5x Phusion GC reaction buffer, 2 μl 2,5 mM deoxyribonucleotide solution, 0.2 μl of Phusion DNA polymerase, and *H. turkmenica* genomic DNA as a template were used. The cycling parameters were as follows: 1. Hot start 95ºC or 98ºC (*Taq or Phusion*, respectively) for 2 min; 2. Denaturation at 95ºC or 98ºC (*Taq* or *Phusion*, respectively), 30 s; 3. Annealing at 55ºC, 1 min; 4. Elongation at 72ºC, 2 min. 30 cycles. 5. Final elongation at 72ºC for 7 min. Purified polynucleotide fragments HTU-AA and HTU-QV were digested with *Bam*H I and *Hin*d III restriction enzymes and ligated into the corresponding sites of the pQE-30 vector (Qiagen), followed by transformation of the *E. coli* strain XL1-Blue by electroporation. Colonies screening was performed by PCR, and the sequence was confirmed by Sanger sequencing.

Protein expression. The XL-1 Blue transformants were grown in LB medium containing ampicillin on an orbital shaker at 37ºC until OD_{600} = 0.7 was reached, followed by induction of expression with IPTG for 3 hours. The cells were then harvested by centrifugation and stored at -70ºC. His-tagged proteins were purified under denaturing conditions (8 M urea) on the metal-chelating sorbent Ni-NTA agarose (*Korneenko and Pestov, 1997*). Typical yields of the purified proteins HTU-QV and HTU-AA were around 25-27 mg per liter of culture. The resulting proteins in 8M urea pH 6.3 buffered with 0.5 M imidazole, 0.1 M sodium phosphate and 20 mM Tris were dialyzed against different buffers (optimization briefly described in Results). The final "optimal" conditions for refolding are as follows:

- Dialysis against 40 mM sodium acetate, pH 6.2 with 1 mM $CuSO_4$ at 4ºC for 3 hours;
- Dialysis against 40 mM sodium acetate, pH 6.2 without copper at 4ºC overnight.

Activity assays. Determination of substrate specificity was performed using a fluorometric method suitable for various amine oxidases as the release of hydrogen peroxide coupled to the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (Biotium, Germany), also known as Amplex red, in the presence of horseradish peroxidase (*Palamakumbura and Trackman, 2002*). The fluorescence of the reaction product (resorufin) was assayed with a Microplate analyzer "Fusion" (Perkin Elmer, USA) at excitation and emission of 535 and 620 nm, respectively. More specifically, the reaction was carried out in 0.1 M borate buffer pH 8.3 in the presence of 1 U / ml horseradish peroxidase. For the negative control, 0.1 mM β-aminopropionitrile was added to block any lysyl oxidase activity. Sheep LOX was isolated from aorta as described before (*Pestov et al, 2014*).

Immunization of rabbits was carried out with purified folded protein HTU-QV. Initially, rabbits were subcutaneously injected with 100 μg protein as an emulsion in Freund's complete adjuvant. The first booster injection was made with the same quantity of the antigen in incomplete Freund's adjuvant 5 weeks after the first immunization, and the second booster injection – with 250 μg antigen and no adjuvants 6 weeks later. One week after the second booster injection sera were collected and stored with the preservative sodium azide at 4ºC. Immunization of rabbits has been approved by Animal Care and Use Review Board of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, protocol No 15/2011.

Western blotting. Following SDS PAGE electrophoresis, *H. turkmenica* protein samples and colored protein weight markers (Pageruler Plus, Thermo, USA) were transferred from polyacrylamide gel onto a PVDF membrane. The membrane was then blocked in liquid non-fat Valio milk with 0.02% sodium azide overnight at 4ºC in order to suppress nonspecific adsorption. On the next stage, the membrane was incubated in 10 ml of TBST buffer solution with primary rabbit antibodies (1:6000) for one hour at room temperature, rinsed out with TBST 4 times for 5 minutes each, followed by incubation with secondary antibodies (HRP-conjugated anti-rabbit antibodies, Biotium, Germany, 1:30000) for one hour in 10 ml TBST, and rinsed out again in the same way. Chemiluminescence was recorded using Femto Maximum Sensitivity Western Blotting Detection Reagent (Thermo, USA) and Fuji X-ray film (Fuji, Japan).

RESULTS

We initially attempted to produce the full-length HTU-LOX protein in *E. coli* but found that it precipitates as inclusion bodies without any detectable amine oxidase activity, and all attempts at its refolding were unsuccessful (results not shown). For this reason, we proceeded to deletion mutants with subsequent purification under denaturing conditions and refolding. The purity of the resulting eluate was checked by SDS PAGE. Fig. 1 illustrates the expression and purification of HTU-LOX exemplified by HTU-QV variant. Of note is the fact of its anomalously slow electrophoretic mobility that corresponds to an apparent molecular weight of 34 kDa, whereas the theoretical value of the His-tagged HTU-QV is 24,3 kDa. Since HTU-LOX is a rather acidic protein (theoretical pI 4.58 for the His-tagged HTU-QV), this peculiarity should be considered as natural.

Refolding of the purified proteins HTU-AA and HTU-QV may be easily achieved using dialysis against different buffers and results in good amine oxidase activity. We investigated a variety of factors that may improve the formation of catalytically active proteins HTU-AA and HTU-QV: buffer type and concentration (Tris, phosphate buffered saline, acetate, etc.), the ionic strength of the solution (concentration of NaCl), temperature, the effect of metals (Cu, Fe, Zn, Ni, Co, Mn) in different concentrations, pH of the solution (5.0-8.0), as well as the dialysis with a gradual decrease in the concentration of the denaturing agent (urea). Optimal pH is around 6.2 (Fig. 2A). It is known that human LOX requires the presence of a copper ion in the catalytic domain in order to achieve the formation of the TPQ cofactor in the catalytic center. For HTU-LOX,

177 indeed, only Cu^{2+} increases activity (Fig. 2B,C), whereas a mixture of different ions gives an
178 inhibition (Fig. 2C). It is interesting to note that although *Haloterrigena turkmenica* is an
179 extreme halophile that requires at least 2 M NaCl, refolding efficiency is not significantly
180 affected by NaCl concentration (Fig. 2B).

181 A slow decrease of the denaturant (urea) concentration was found to lack any advantages over
182 the stepwise approach with immediate transfer into a buffer without urea. This was confirmed by
183 refolding by dilution (results not shown). Ultimately, a simple refolding procedure may be
184 considered as optimal: firstly, the purified denatured HTU-LOX protein is dialyzed for a few
185 hours against acetate buffer (pH 6.2) with Cu^{2+}, followed by overnight dialysis against the same
186 buffer without copper, in the cold.

187 Interestingly, under any conditions used, the amine oxidase activity of the protein HTU-QV was
188 about fifteen times higher than that of HTU-AA. Therefore, the segment of HTU-LOX sequence
189 from Ala^{39} to Gln^{92} may function as an inhibitory (pro)peptide (results not shown).

190 Refolded proteins HTU-QV and HTU-AA exhibit activity against a wide variety of primary
191 amines (Table): histamine, methylamine, lysine, cadaverine, tyramine etc. Even glycine, β-
192 alanine and taurine are efficiently oxidized, in contrast with mammalian LOX. HTU-LOX
193 readily oxidizes some amine-containing antibiotics: polymyxin and aminoglycosides such as
194 capreomycin and amikacin. This is a unique property of lysyl oxidases, since other amine
195 oxidases either do not deaminate aminoglycosides or even are inhibited by them, as in the case of
196 ECAO (Elovaara et al, 2015). Regarding various proteins, lysine-containing peptides and
197 polymers (e.g., poly-L-lysine, poly-allylamine, lysozyme, and substance P as an example of a
198 Lys-containing peptide), the HTU-LOX behaves almost like LOX from the aorta. Taurine is one
199 of the best substrates for HTU-LOX. It is also capable of oxidizing glycine, β-alanine, and γ-
200 aminobutyric acid. This observation is unusual, because the presence of any acidic groups in
201 vicinity of the amino group almost completely prevents oxidation by most amine oxidases. Thus,
202 it is safe to conclude that the HTU-LOX has a relaxed substrate specificity in comparison with its
203 mammalian homologue (*Shah et al, 1993*). The only amine that HTU-LOX oxidizes much worse
204 than mammalian LOX is benzylamine. Importantly, the HTU-LOX demonstrated good
205 sensitivity to the classical inhibitor of all LOXes - β-aminopropionitrile (BAPN). Also, HTU-
206 LOX is somewhat different from the mammalian enzyme in terms of pH dependence. In contrast

to the latter, HTU-LOX activity does not exhibit a steep decline from its maximum around 8.3, and even displays a certain degree of bimodality retaining some activity even below 7 (Fig. 3).

We also attempted to study the HTU-LOX protein in the host - the archaeal halophile *H. turkmenica*. For this purpose, we raised polyclonal antibodies against the truncated HTU-LOX (variant QV) and used them for detection of the original protein in *H. turkmenica*. Western blot (Fig. 4, lanes 1-3) showed the presence of the protein in the cell lysate. It should be emphasized that this band was reliably detected only at a high sensitivity, meaning that the normal expression level of the protein in cultured *H. turkmenica* is quite low. None HTU-LOX has been detected among secreted proteins of the culture medium. The full-size *H. turkmenica* lysyl oxidase theoretically contains 308 amino acids with a molecular weight of 33829 Da, whereas the detected protein band has electrophoretic mobility corresponding to molecular weight of 49 kDa. Therefore, *H. turkmenica* contains small quantities of a LOX gene expression product that most likely represents a full-length, unprocessed protein. We tried to find any products that may become active after a proteolytic cleavage. Samples of *H. turkmenica* at different stages of culture growth (including a long-term storage for a year), as well as other parameters like the presence or absence of light, salinity, concentration of copper and presence of antibiotics were analyzed, but no reliable effects of these conditions on the expression level of HTU-LOX have been demonstrated (results not shown). However, growth without yeast extract resulted in formation of a band with a molecular weight decreased by about 3 kDa (Fig. 4 lane 5). Also, we found that BAPN (even at a rather high concentration of 1 mM) had no significant effect on sensitivity of fresh cells to osmotic stress, on formation of hypotonically-resistant cysts, or on growth rate in both conventional (IAO) and defined (MHTU) media.

DISCUSSION

Amino acid sequence alignments (Fig. 5-7) of LOX proteins demonstrate poor overall conservation (for example, high variability in the number of disulfide bonds) with only a few hyperconserved amino acid residues like Cu-binding His and TPQ formation. The apparent phylogenetic tree of LOX catalytic domain (Fig. 8) tempts to speculate that LOX originated from actinomycetes, and then spread to other branches of prokaryotes, as well as into an ancestor of the modern animal kingdom. Also, LOX genes were repeatedly lost, for example, in ctenophores and nematodes. Of course, many other scenarios are possible, since all these events of HGT are

ancient. Another fundamental aspect that needs to be emphasized is the fact that relatively little research has been carried out on the influence of HGT with the subsequent adaptation of the catalytic properties of the enzymes to a new host.

Refolding efficiency is not significantly affected by NaCl concentration. This surprising fact could reflect the history of prokaryote LOX genes: halophile archaea may have acquired these genes from microorganisms with a rather different requirement for salt. The ancient HGT event may had even originated from a halophobic organism, followed by "domestication" that suppressed the formation of misfolded protein. Besides, LOX may had served as an antibiotic resistance enzyme under aerobic conditions. This, however, is unlikely in extant *H. turkmenica*, since Archaea are usually highly resistant to both polymyxin and common aminoglycosides. Also, HTU-LOX oxidizes some peptide antibiotics and theoretically this feature may be useful for competition with other species of haloarchaea (*Besse et al, 2015*) in the natural habitat of *H. turkmenica*. The low expression level of the enzyme suggests that HTU-LOX plays a modest functional role in increasing availability of nitrogen from non-typical amines. Its promiscuous substrate specificity and negligible enzymatic activity in *H. turkmenica* cells make it difficult to demonstrate this fact experimentally.

Another interesting fact is that the amine oxidase activity of the truncated protein HTU-QV is much higher than that of the longer one, HTU-AA. This observation is in line with the general view that LOX catalytic domain is always (except for animal LOXL2-4 and homologs) preceded by an autoinhibitory sequence, together forming a propeptide. In the case of HTU-LOX, the autoinhibitory sequence corresponds to the stretch from Ala^{39} to Gln^{92}. However, the inhibition is relatively inefficient, and this may also reflect the evolution of HTU-LOX gene after the in-Archaea HGT that resulted in a partial degradation of the autoinhibitory function of the propeptide.

What is the origin of the animal lysyl oxidase? Has it emerged in primitive animals at the beginning of their evolution from Eubacteria? Or, conversely, LOX genes, which have important functions in animals, made their way several times into the world of prokaryotes? Although the second option is less likely due to splitting of animal ORFs into exons, it allows a more parsimonious explanation of the evolution of the catalytic LOX domain both in animals and

bacteria. The only obvious fact is that inter-kingdom saltations of LOX genes between distant branches of Life occurred more than once.

CONCLUSIONS

- *H. turkmenica* LOX (HTU-LOX) may be successfully expressed in *E. coli*
- Optimal refolding conditions is different from those for the growth of the host cells
- Sensitivity to β-aminopropionitrile is conserved in HTU-LOX
- HTU-LOX has a relaxed substrate specificity in comparison with mammalian LOX
- Benzylamine is a poor substrate for HTU-LOX
- N-terminal truncation of HTU-LOX increases activity
- Cultured *H. turkmenica* does not exhibit any detectable amine oxidase activity
- In *H. turkmenica,* the HTU-LOX is present at low levels in an unprocessed form
- Native *H. turkmenica* lysyl oxidase function may be cryptic

ACKNOWLEDGMENTS

These studies were supported by MCB RAS and Russian Foundation for Basic Research (grant 16-04-01755), experiments with antibiotics were supported by Russian Science Foundation (grant No 16-14-10335).

REFERENCES

Besse A, Peduzzi J, Rebuffat S, Carré-Mlouka A. 2015. Antimicrobial peptides and proteins in the face of extremes: Lessons from archaeocins. *Biochimie* **118: 344-355.**

Grau-Bové X, Ruiz-Trillo I, Rodriguez-Pascual F. 2015. Origin and evolution of lysyl oxidases. *Scientific Reports* **5:** 10568

Elovaara H, Huusko T, Maksimow M, Elima K, Yegutkin GG, Skurnik M, Dobrindt U, Siitonen A, McPherson MJ, Salmi M, Jalkanen S. 2015. Primary amine oxidase of *Escherichia coli* is a metabolic enzyme that can use a human leukocyte molecule as a substrate. *PLOS One* **10:** e0142367

Korneenko TV, Pestov NB, Egorov MB, Ivanova MV, Kostina MB, Shakhparonov MI. 1997. Monoclonal antibodies to the α-subunit of the putative human H^+,K^+-ATPase encoded by the atp1al1 gene. *Bioorganicheskaya khimiya* 23: 800-8004.

Mosin O., Ignatov, I. 2014. Photochrome Transmembrane Protein Bacteriorhodopsin from Purple Membranes of *Halobacterium halobium*. Applications in Bio- and Nanotechnologies. *Journal of Medicine, Physiology and Biophysics* **6:** 42-60

Noda-García L, Camacho-Zarco AR, Medina-Ruíz S, Gaytán P, Carrillo-Tripp M, Fülöp V, Barona-Gómez F. 2013. Evolution of substrate specificity in a recipient's enzyme following horizontal gene transfer. *Molecular Biology and Evolution* **30:** 2024-2034

Palamakumbura AH, Trackman PC. 2002. A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. *Analytical Biochemistry* **300:** 245-251.

Papke RT, Corral P, Ram-Mohan N, Haba RR, Sánchez-Porro C, Makkay A, Ventosa A. 2015. Horizontal gene transfer, dispersal and haloarchaeal speciation. *Life (Basel)* 5: 1405-1426

Pestov NB, Okkelman IA, Shmanai VV, Hurski AL, Giaccia AJ, Shchepinov MS. 2011. Control of lysyl oxidase activity through site-specific deuteration of lysine. *Bioorganic & Medicinal Chemistry Letters* **21:** 255-258.

Saunders E, Tindall BJ, Fähnrich R, Lapidus A, Copeland A, Del Rio TG, Lucas S, Chen F, Tice H, Cheng JF, Han C, Detter JC, Bruce D, Goodwin L, Chain P, Pitluck S, Pati A, Ivanova N, Mavromatis K, Chen A, Palaniappan K, Land M, Hauser L, Chang YJ, Jeffries CD, Brettin T, Rohde M, Göker M, Bristow J, Eisen JA, Markowitz V, Hugenholtz P, Klenk HP, Kyrpides NC. 2010. Complete genome sequence of *Haloterrigena turkmenica* type strain (4k). *Standards in Genomic Sciences* **2:** 107-116

Shah MA, Scaman CH, Palcic MM, Kagan HM. 1993. Kinetics and stereospecificity of the lysyl oxidase reaction. *Journal of Biological Chemistry* 268: 11573-11579

Ventosa A, Carmen Gutierrez M, Kamekura M, Dyall-Smith M L. 1999. Proposal to transfer *Halococcus turkmenicus*, *Halobacterium trapanicum* JCM 9743 and strain GSL-11 to *Haloterrigena turkmenica* gen. nov., comb. nov. *International Journal of Systematic Bacteriology* **49:** 131-136

Zvyagintseva IS, Tarasov AL. 1987. Extreme halophilic bacteria from alkaline soils. *Mikrobiologiya* **56:** 839-844

FIGURE LEGENDS

TABLE

Substrate efficiencies of recombinant *Haloterrigena* lysyl oxidase (HTU-QV variant) in comparison with mammalian enzymes from aorta. V_{max}/K_m ratios normalized with respect to tyramine. Values for polymers and lysozyme calculated per amine groups. Data for LOX from bovine aorta from (*Shah et al, 1993*) (note that conditions used were significantly different from this study). For HTU-LOX the short variant QV has been used. ND – no data. Z – rate too low for accurate determination. CK – complex kinetics with inhibition by substrate at high concentrations.

Figure 1. Electrophoretic analysis of recombinant N-terminally truncated *H. turkmenica* LOX (HTU-QV) expression and purification. 1 - molecular weight marker proteins; 2 - *E. coli* proteins before addition of IPTG; 3 – Expression induced with IPTG; 4 – purified HTU-QV protein.

Fig. 2. Folding of recombinant *H. turkmenica* lysyl oxidase (HTU-QV). Folding by dialysis. A – Influence of pH; B – effect of NaCl concentration; C – 1 mM salts of various metals added to the dialysis buffer; D – different concentrations of $CuSO_4$. In the case of salt similar results were obtained in folding by dilution experiments, demonstrating also that 1 M and 2M NaCl cannot improve activity any further (results not shown).

Fig. 3. pH dependence of recombinant *H. turkmenica* lysyl oxidase. In comparison with LOX from sheep aorta amine oxidase reaction rates were measured for HTU-QV protein if universal borate-phosphate-acetate buffer with histamine as the substrate.

Figure 4. Immunoblotting detection of lysyl oxidase in *H. turkmenica* cells with anti-HTU-LOX antibodies. 1,2,3,5 – chemiluminescence of bound HRP-labeled antibodies; 4 – molecular weight markers. The electrophoretic mobility of the detected band corresponds to a molecular weight about 49 kDa. Theoretical value for the full-length HTU-LOX is 32.4 kDa. This

apparently large discrepancy should be regarded as normal, however, since the anomalous mobility has been observed for recombinant HTU-LOX (Fig. 1). 1-3 - *H. turkmenica* cells grown in IAO medium (twofold dilutions from lane 3 to 1), 5 - *H. turkmenica* cells grown in IAO medium without yeast extract. * - non-specific band from binding of HRP-conjugated secondary antibodies to a contamination in reagents.

Fig. 5. Amino acid sequence alignment of the mouse lysyl oxidase (LOX isoform) and *Haloterrigena turkmenica* HTU-LOX (Haloterrigena). Obtained using Muscle algorithm. Cysteine residues putatively involved in disulfide formation marked in yellow, LTQ-forming lysine and tyrosine - red, purple - hyperconserved three histidine residues necessary for the binding of copper (the whole site is underlined). Dark green – hypothetical proteolytic cleavage sites. AA and QV residues of HTU-LOX in bold and the connecting stretch italicized.

Fig. 6. Multiple alignment of archaeal lysyl oxidases. Obtained using Muscle algorithm. (HaloterrigenaturCk – *Haloterrigena turkmenica*, NatronoCoCCusjeotgali – *Natronococcus jeotgali,* HaloterrigenalimiCola –*Haloterrigena limicola*, Nitrosopumilus – *Nitrosopumilus.* Cysteine residues marked in yellow marked, LTQ-forming lysine and tyrosine - red, purple - hyperconserved three histidine residues necessary for the binding of Cu^{2+}.

Fig. 7. Multiple alignment of the conserved segments of catalytic domains from all lysyl oxidases representing different kingdoms. Obtained using Muscle algorithm from consensus sequences of different taxa. ARCHAE – Archaeal LOX sequences, DELTA – *Deltaproteobacteria*, FUNGI – fungal LOXes, ANIMA – various animal LOXes, LOW – *Mesomycetozoa* and *Orthonectida*, short – LOX from *Truepera radiovitrix, Deinococcus pimensis, Nitrospira nitrosa* and a few samples from Parcubacteria*,* BETA – *Betaproteobacteria*, Actinshort – *Amycolatopsis mediterranei* LOX and closest homologues, STREPTOM – LOX from *Streptomyces*, ACTINvar – other actinomycetal LOXes, BACIES – all other eubacterial LOXes. Cysteine residues marked in yellow marked, LTQ-forming lysine and tyrosine - red, purple - hyperconserved three histidine residues necessary for the binding of copper.

Fig. 8. Apparent phylogenetic tree of the catalytic domain of lysyl oxidase. Mouse LOX isoforms (lox and loxl1-4), “drome” - *Drosophila*, SorangiumC – *Sorangium cellulosum*, *S*… - different species of *Streptomyces* (except for Strongylocentrotus и Saccoglossus). Constructed with maximum likelihood approach using PhyML with Approximate Likelihood-Ratio Test at http://www.phylogeny.fr. Sequences used were GenBank entries theoretically predicted from genomes. Suspicious closeness of deltaproteobacteria LOXes (*Myxococcus xanthus* and *Plesiocystis*) to the animal proteins points to the possibility of their origin from a HGT independent from those between actinobacteria and archaea.

Table 1 (on next page)

TABLE Substrate efficiencies of recombinant *Haloterrigena* lysyl oxidase (HTU-QV variant) in comparison with mammalian enzymes from aorta.

V_{max}/K_m ratios normalized with respect to tyramine. Values for polymers and lysozyme calculated per molar amine groups. Data for LOX from bovine aorta from (*Shah et al, 1993*) (note that conditions used were significantly different from this study). For HTU-LOX the short variant QV has been used. ND – no data. Z – rate too low for accurate determination. CK – complex kinetics with inhibition by substrate at high concentrations.

Table

substrate	HTU-LOX	Sheep	Bovine
L-lysine	0.058	0.028	ND
cadaverine	0.370	1.070	1.09
histamine	0.550	0.920	ND
taurine	1.120	0.120	ND
glycine	0.020	z	ND
β-alanine	0.005	z	ND
GABA	0.015	z	ND
methylamine	0.020	ND	ND
substance P	0.068	ND	ND
lysozyme	CK	0.080	ND
polyallylamine	CK	0.080	ND
amikacin	0.260	ND	ND
capreomycin	0.120	0.190	ND
polymyxin	0.780	ND	ND
benzylamine	z	0.170	0.52
hexylamine	0.28	1.140	0.14

Substrate efficiencies of recombinant *Haloterrigena* lysyl oxidase (HTU-QV variant) in comparison with mammalian enzymes from aorta. V_{max}/K_m ratios normalized with respect to tyramine. Values for polymers and lysozyme calculated as for molar amine groups. Data for LOX from bovine aorta from (*Shah et al, 1993*) (note that conditions used were significantly different from this study). For HTU-LOX the short variant QV has been used. ND – no data. Z – rate too low for accurate determination. CK – complex kinetics with inhibition by substrate at high concentrations.

Figure 1

Fig. 1. Electrophoretic analysis of recombinant N-terminally truncated *H. turkmenica* LOX (HTU-QV) expression and purification.

1 - molecular weight marker proteins; 2 - *E. coli* proteins before addition of IPTG; 3 – Expression induced with IPTG; 4 – purified HTU-QV protein.

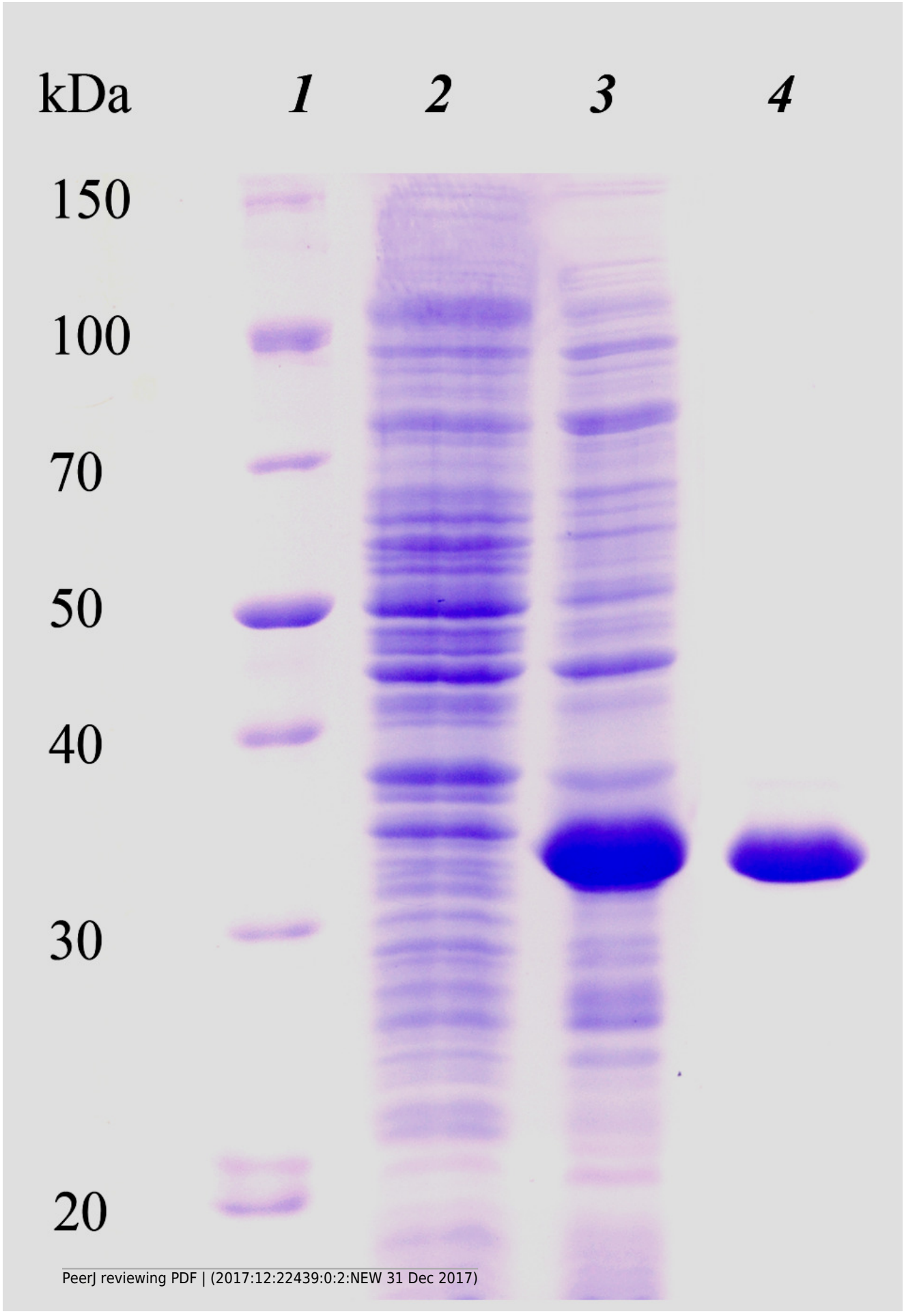

kDa
1
2
3
4
150
100
70
50
40
30
20

Figure 2

Fig. 2. Folding of recombinant *H. turkmenica* lysyl oxidase (HTU-QV).

Folding by dialysis. A – Influence of pH; B – effect of NaCl concentration; C – 1 mM salts of various metals were added to the dialysis buffer; D – different concentrations of CuSO4. In the case of salt similar results were obtained in folding by dilution experiments, demonstrating also that 1 M and 2M NaCl cannot improve activity any further (results not shown).

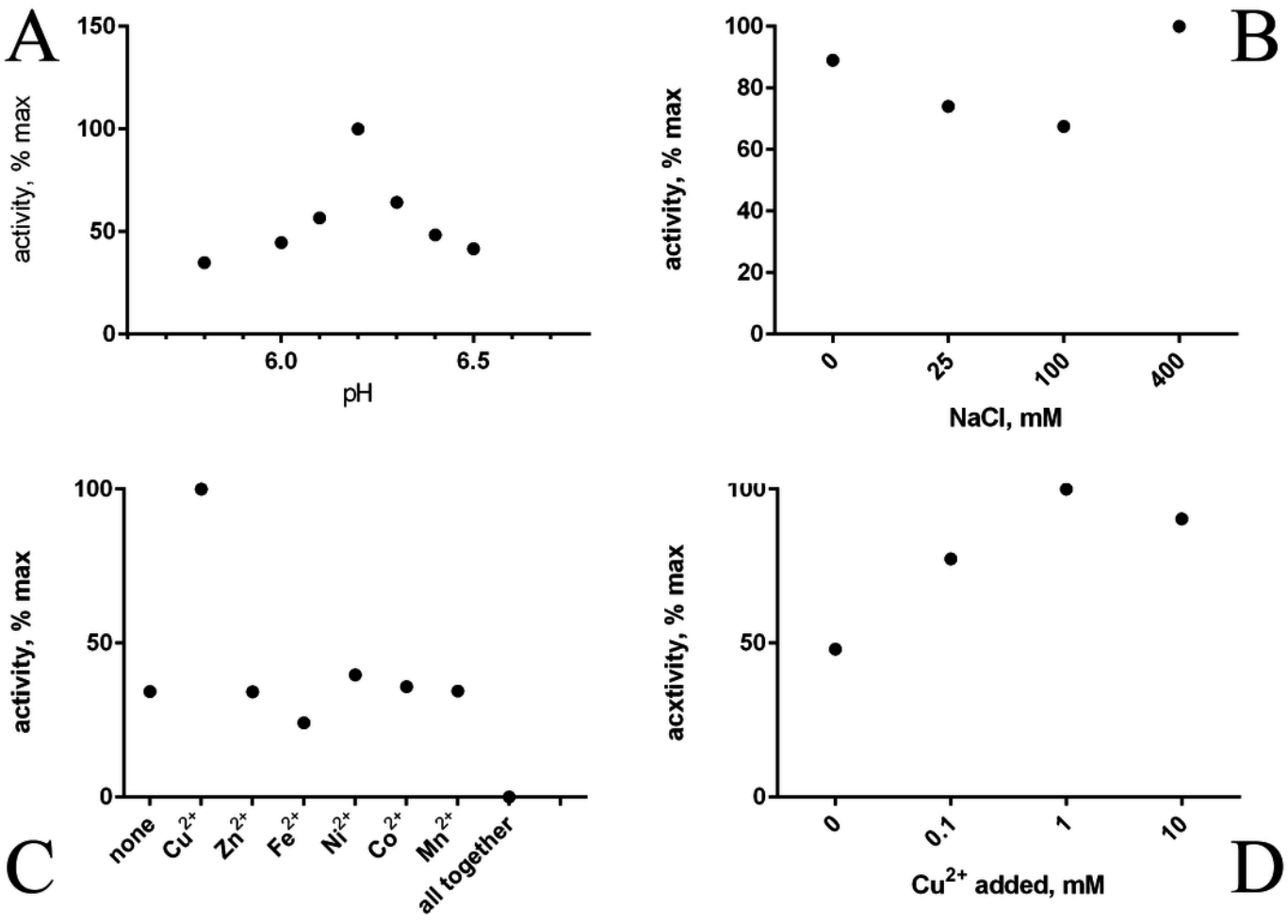

A
activity, % max
150
100
50
0
6.0
6.5
pH
B
activity, % max
100
80
60
40
20
0
0
25
100
400
NaCl, mM
C
activity, % max
100
50
0
none
Cu2+
Zn2+
Fe2+
Ni2+
Co2+
Mn2+
all together
D
acxtivity, % max
100
50
0
0
0.1
1
10
Cu2+ added, mM

Figure 3

Fig. 3. Fig. 3. pH dependence of recombinant *H. turkmenica* lysyl oxidase.

In comparison with LOX from sheep aorta amine oxidase reaction rates were measured for HTU-QV protein if universal borate-phosphate-acetate buffer with histamine as the substrate.

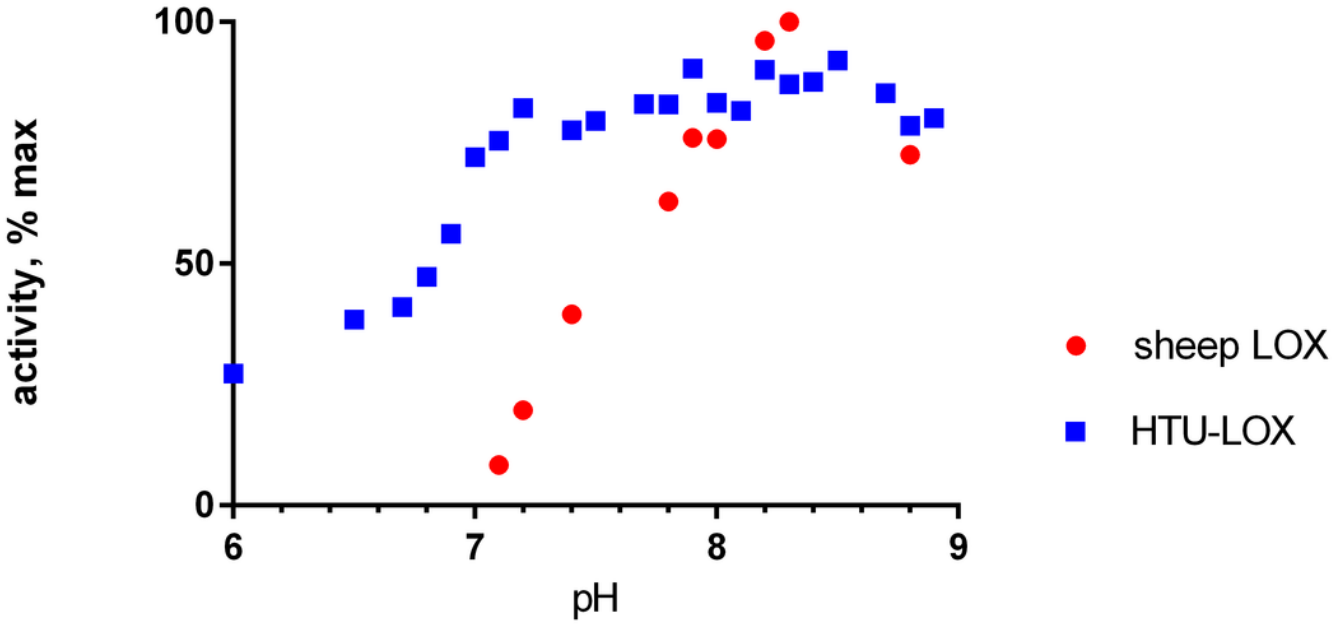


Figure 4

Figure 4. Immunoblotting detection of lysyl oxidase in *H. turkmenica* cells with anti-HTU-LOX antibodies.

1,2,3,5 – chemiluminescence of bound HRP-labeled antibodies; 4 – molecular weight markers. The electrophoretic mobility of the detected band corresponds to a molecular weight about 49 kDa. Theoretical value for the full-length HTU-LOX is 32.4 kDa. This apparently large discrepancy should be regarded as normal, however, since the anomalous mobility has been observed for recombinant HTU-LOX (Fig. 1). 1-3 - *H. turkmenica* cells grown in IAO medium (twofold dilutions from lane 3 to 1), 5 - *H. turkmenica* cells grown in IAO medium without yeast extract. * - non-specific band from binding of HRP-conjugated secondary antibodies to a contamination in reagents.

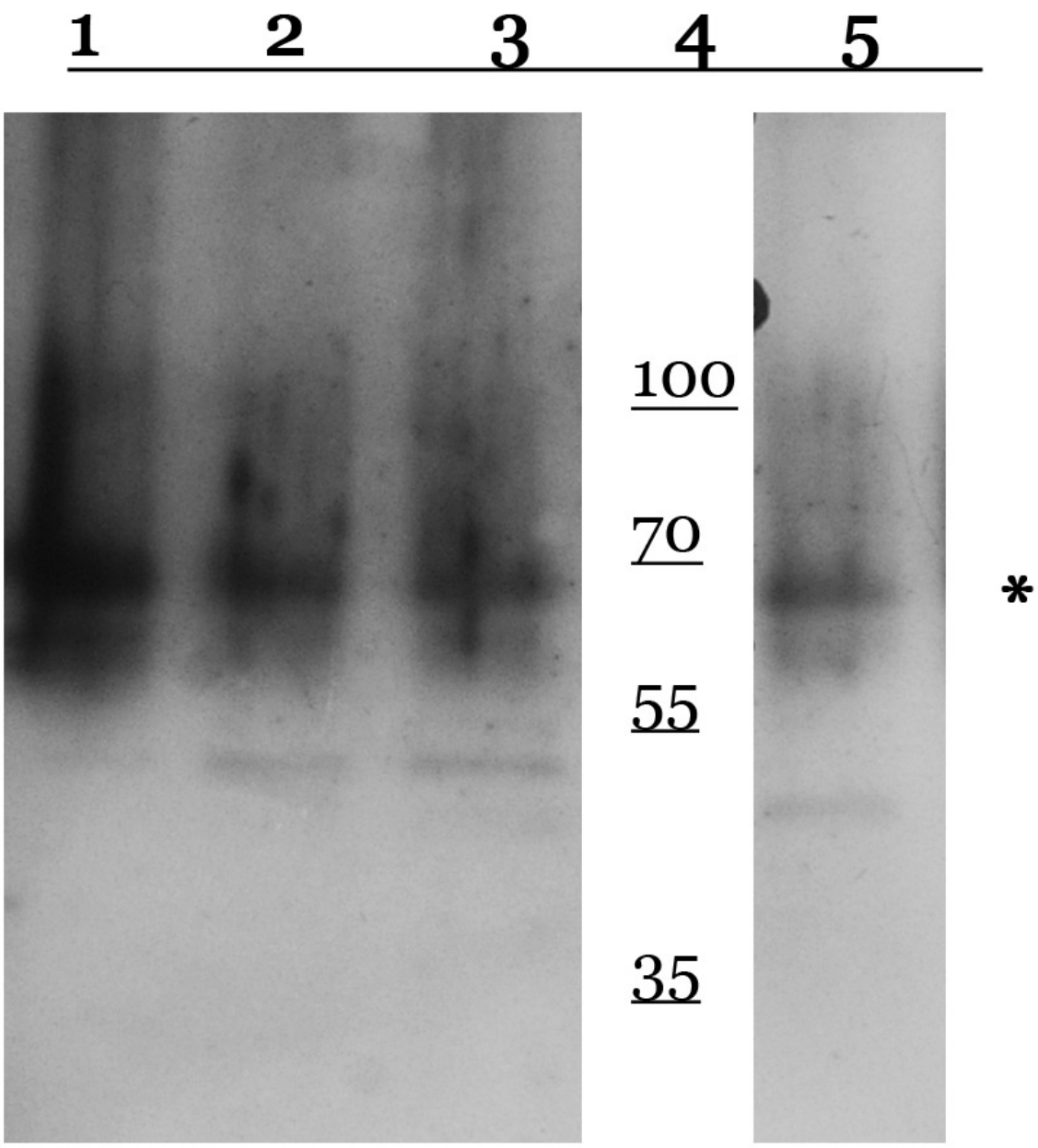

1
2
3
4
5
100
70
55
35
*

Figure 5

Fig. 5. Amino acid sequence alignment of the mouse lysyl oxidase (LOX isoform) and *Haloterrigena turkmenica* HTU-LOX (Haloterrigena).

Obtained using Muscle algorithm. Cysteine residues putatively involved in disulfide formation marked in yellow, LTQ-forming lysine and tyrosine - red, , purple - hyperconserved three histidine residues necessary for the binding of copper (the whole site is underlined). Dark green – hypothetical proteolytic cleavage sites. AA and QV residues of HTU-LOX in bold and the connecting stretch italicized.

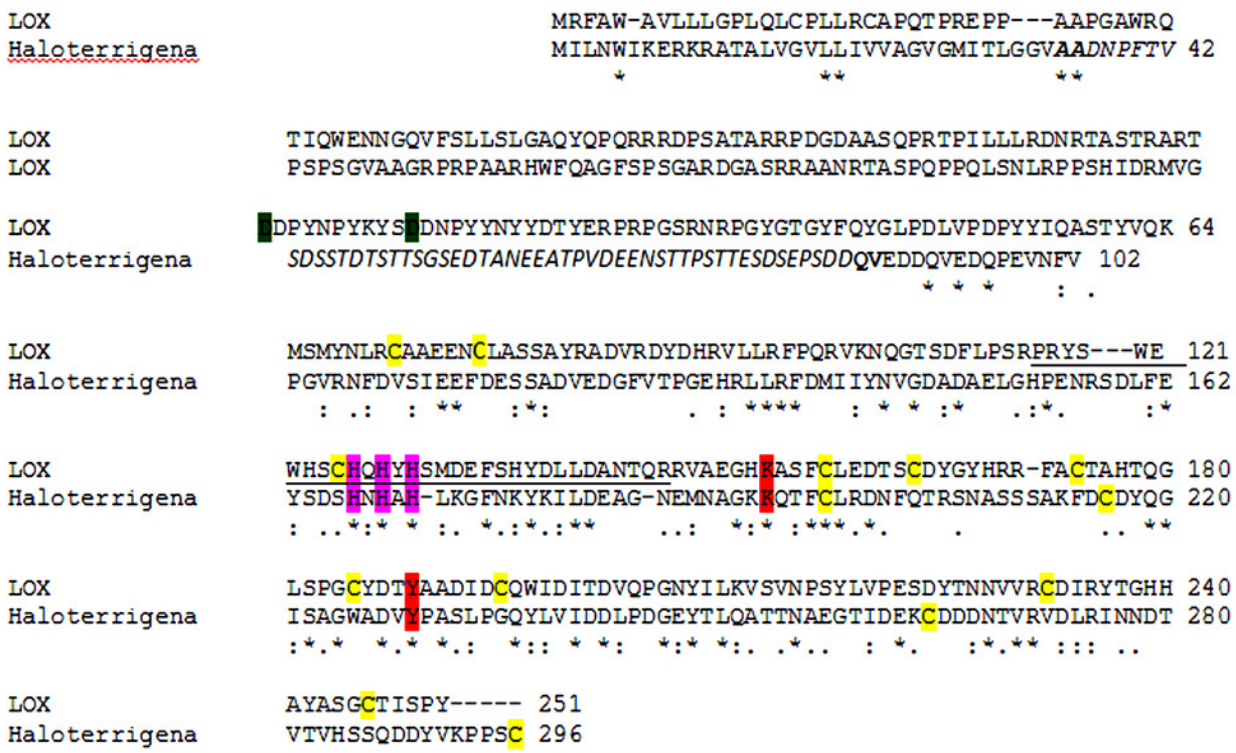


Figure 6

Fig. 6. Multiple alignment of archeal lysyl oxidases.

Obtained using Muscle algorithm. (HaloterrigenaturCk – *Haloterrigena turkmenica*, NatronoCoCCusjeotgali – *Natronococcus jeotgali,* HaloterrigenalimiCola –*Haloterrigena limicola*, Nitrosopumilus – *Nitrosopumilus.* Cysteine residues marked in yellow marked, LTQ-forming lysine and tyrosine - red, purple - hyperconserved three histidine residues necessary for the binding of Cu^{2+}.

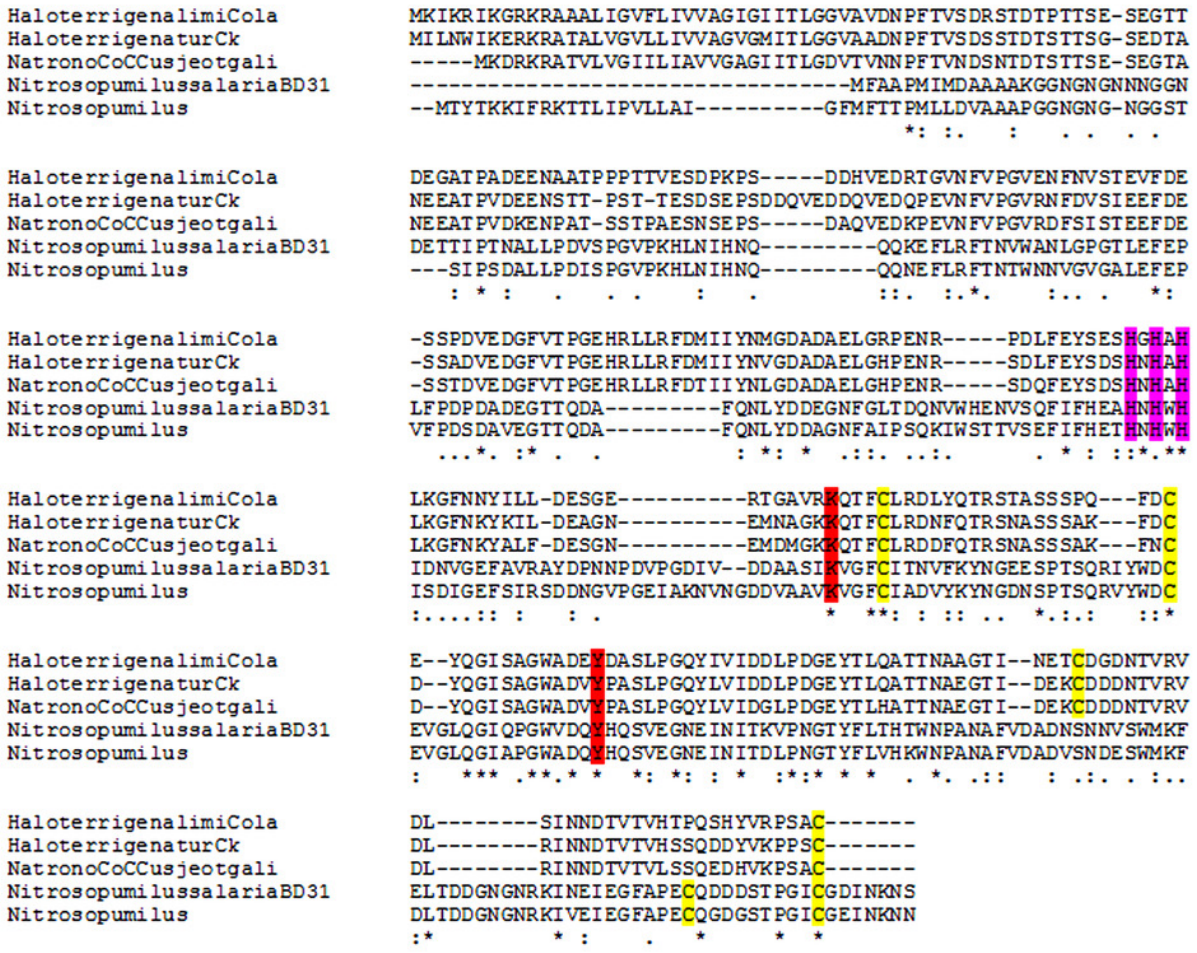


Figure 7

Fig. 7. Multiple alignment of the conserved segments of catalytic domains from all lysyl oxidases representing different kingdoms.

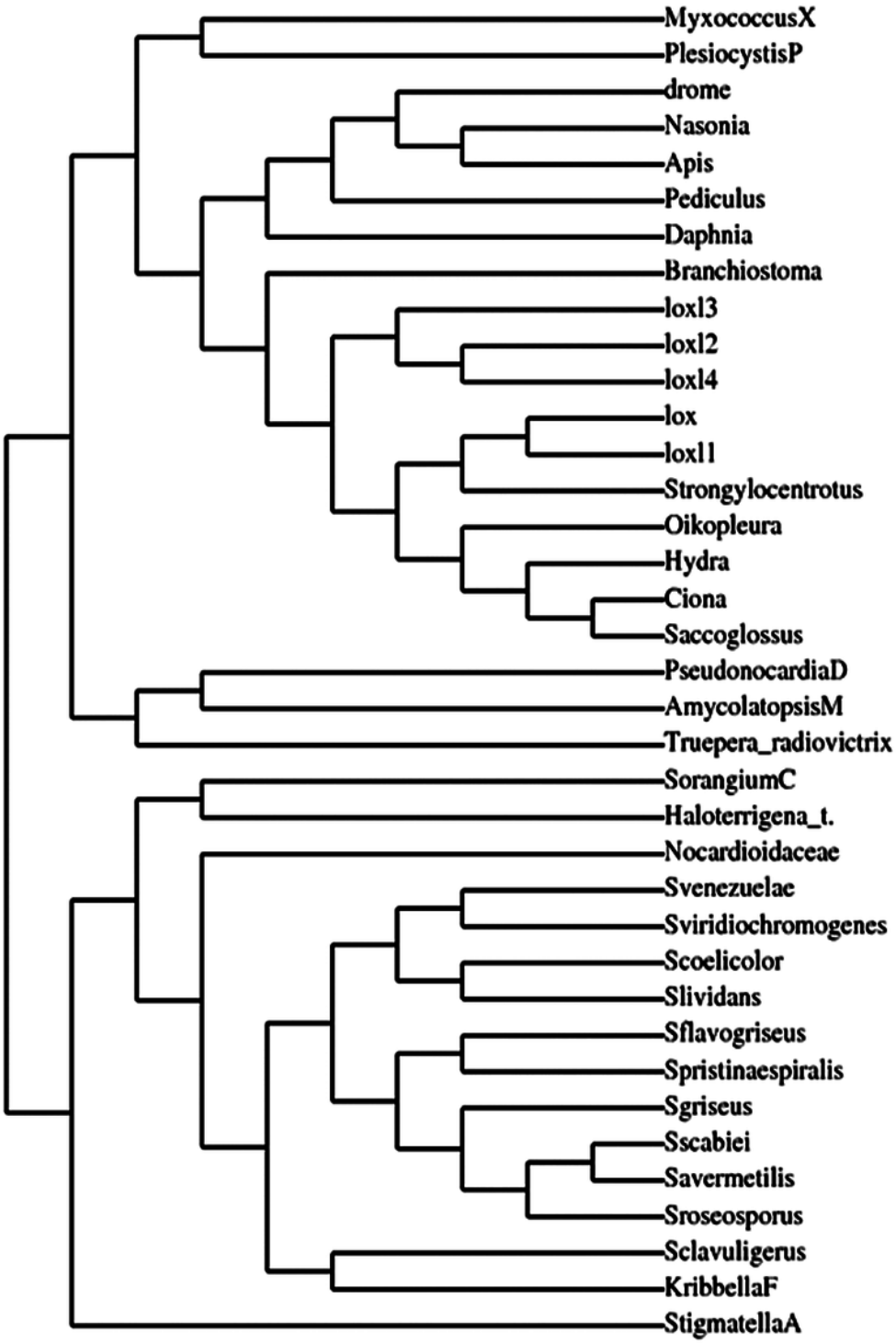
Obtained using Muscle algorithm from consensus sequences of different taxa. ARCHAE – Archaeal LOX sequences, DELTA – *Deltaproteobacteria*, FUNGI – fungal LOXes, ANIMA – various animal LOXes, LOW – *Mesomycetozoa* and *Orthonectida*, short – LOX from *Truepera radiovitrix, Deinococcus pimensis, Nitrospira nitrosa* and a few samples from Parcubacteria, BETA – *Betaproteobacteria*, Actinshort – *Amycolatopsis mediterranei* LOX and closest homologues, STREPTOM – LOX from *Streptomyces*, ACTINvar – other actinomycetal LOXes, BACIES – all other eubacterial LOXes. Cysteine residues marked in yellow marked, LTQ-forming lysine and tyrosine - red, purple - hyperconserved three histidine residues necessary for the binding of copper.

```
ARCHEA          -------KESNSEPSDDQVEDKPEVNFVPGVRNFGVSTEEFDESSPDVEDGFVTPGEHRL
DELTA           -------------VDADVISRTVYIERRTFAADACEVYEGCVGAPGR----------RRL
FUNGI           -------------DADWLQKHLYIDYVDAAEDPCLINEGCLTGPPA-------------
ANIMA           -------------MDALLVQQTAHLEDRPLYLLGCAMEENCLASSAYQVEPGWPYGTRRL
LOW             -------------MNSNNAQSTLVLSAGHLYNTQCAMEEGCLASGAW----------RKL
BACIES          -------------QCPPGTNCELLPDLVILPRFTRSQIKEYSNDDPY---------YGGQ
short           --------------------PNRLLPDLVIYPPSELSIVGSEKTG------------RRE
BETA            ------------------ATTNRLPNLKPLPASNLSLVADSAGGST--------------
ACTINshort      ----------------------LPDLRQAPIGDLQVQTG-----PS---------GQVR
ACTINvar        ------------------------------------------AK----------AVR
STREPTOM        QAPAPALKANAKRPTKATVPNVPKPDLRSLPAYGITVSDGYEDVPG----------KDY

ARCHEA          LRFDMIIYNLG-DADAELGRPE-----------------------------------N
DELTA           LRFSVSIPNLG-SAAVIPPPE-----------------------------------E
FUNGI           ----------DRDDP-------------------------------------------N
ANIMA           LRFTARIWNRG-TADFLPK---------------------------------------R
LOW             LRFSASFWNFG-TADFLP----------------------------------------N
BACIES          LRFAATIANIG-DGPMETRGYCGTLGVVSNSICPDGSYPRQVLFQRIYSLKDKNLSSVDR
short           IKFATTVWNIGKSGPLELIGTV------------DPATNKTRVYQRIKNRGGESAS---R
BETA            LRFNTTSWNKG-SGPLVLGAGA------------VDTGSGKQQVFQRVFLSNGGYFV---P
ACTINshort      LRFTTSIVNV--DGPLLLVAHR------------DSTDVPFMAVQAI-QSDGSIADV--E
ACTINvar        LRFTAAEWNAG-DGPLLLYGRR------------DSATDTMDVRQYFFDAKHGQVQR--Q
STREPTOM        LAFSANVWNAG-PAKLVVDGFR------------SPGKELMDAYQYFYDAKGKQVGY--T

ARCHEA          RPDQFEYSES- HGHAH-LKGFNKYAI--LDESGN---EMNAGKKQTFCLRDVFQTRS--
DELTA           NPDLYVYDEC--HQHEH-LVNFASYEL--RDADNN---VVAVGRKQGFYLVD-MEPYC--
FUNGI           NPPYWHWDTC--HEHWH-FTAYANYRL--LSANGS--EVVAQGHKNGFCLED-SLCDE--
ANIMA           PRHSWEWHAC--HQHYHSMEVFAHYDL--LDLNGT---KVAEGHKASFCLED-TECDG--
LOW             PDDGPEWHEC--HNHYH-ISNFANYTI--TGSAGN---QLTQGHKQSFCLED-VKCLP--
BACIES          PAGTNYYDNQPGHNHYH-VDDWVEFRLVKIEP-GKRASIIAKGRKVSYCLFDSGICNNAD
short           TAGYFEYHPD--HEHWHLFNDFATYELWTLNADGSLETLVATSGKVTFCLMDTTAVDP--
BETA            VAGGFEWHPA--HNHFH-FDDFALYTLQPVNAPGG---VVRTGSKTTFCLMDSTRIDS--
ACTINshort      TPASLYYEPADGHDHWH-LLDFEYYQL--RRPDGG---VVVTDRKNGFCIGDRYRVRD--
ACTINvar        TAGTMYYEPAPMHQHWH-LLDFARYQL--RTPDGE---TVVRDRKNGFCLADRYADVD--
STREPTOM        PTGTMEWDPRPGHEHWH-FTDFASYRL--LKADKK---ETVRSGKEAFCLANTDAVD---

ARCHEA          ------NASSS----------AKFDCE-------YQGISAGWADVYP-ASLPGQYLVI-D
DELTA           ------DAAPR----------AYTCG-------GQGISPGWSDTYA-ADTPCQWLDV-T
FUNGI           ------GVAP-----------FYNCT-------NQGITMGCHDLYD-AGLGCQWIDI-T
ANIMA           ------GVQRR---------   -YCANYG----DQGISVNCWDTYR-HDIDCQWIDI-T
LOW             ------SLLPK-----------YICN-------NQGISVGCADSYSVSNIDCQWIDI-T
BACIES          SLCTINGTVYGERNLSNYGLGNYASCN-----AMKQGISVGGYDTYG-VMYEGQFLQLPK
short           ------YPLPN---APGGP--TYSSCG-----NNVQGISVGWGDTYG-AKLAGQEIDL-T
BETA            -------SLPG---APGQA--VYSTCG-----RTIGGISVGWGDTYG-AHLPGQEIDF-T
ACTINshort      -------DLPG---RPADPYVLGHMCG-PAALTVKMGISVGWGDDYK-HTLPFQWLDI-T
ACTINvar        ------YTLPNAVWRPENT-DLATSCGDPSSLDVREGISVGS-DDYR-YTVDFQWLDI-T
STREPTOM        ------YTVKNANWHPDNT-DLSTACGQENSISVREVLDVGSGDTYT-QDLPGQSFDI-T

ARCHEA          DL-------PDGEYTLQATTNAEGTIDEKCDDDNTVRVDLRI----------------
DELTA           DV-------PDGTYTLRVGVDTRDIVDEGDVHPNTVDVPVR----------------
FUNGI           DLHLQPGYSPNTEYTLSVILNPEKAIPETDYSNNAAV--------------------
ANIMA           DV-------PPGNYILKVVVNPEFAVAESDFTNNAVRCNIRY----------------
LOW             PL-------KSGWYVLNVYVNPDKRVTESDYTNNVFHVLFRF----------------
BACIES          GL-------ASGTYILEIE-DPTGSFYEKNRSNNLFRMPIVIEKQ--------------
short           DV-------PDGRYLLRVEVDPEDRIEELDYDNNFSVTFVEI----------------
BETA            GN-------ADGTYQLRIVIDPNKVIIESDESNNASCVLISIRKPNTVTVLDSSGSCSTA
ACTINshort      GL-------PAGRYDLVNVADPDGALLEKNYDNNASWVDISVTSP--------------
ACTINvar        HV-------PSGTYDLVNTVNPDRTL-ETSYDNNSSSIAIVLGGT--------------
STREPTOM        GL-------PNGTYYIQVLANPENRLKETNHKNNSALRKVVLGGK--------------
```

Figure 8

Fig. 8. Apparent phylogenetic tree of the catalytic domain of lysyl oxidase.

Mouse LOX isoforms (lox and loxl1-4), “drome” - *Drosophila*, SorangiumC – *Sorangium cellulosum*, S... - different species of Streptomyces (except for Strongylocentrotus и Saccoglossus). Constructed with maximum likelihood approach using PhyML with Approximate Likelihood-Ratio Test at http://www.phylogeny.fr. Sequences used were GenBank entries theoretically predicted from genomes. Suspicious closeness of deltaproteobacteria LOXes (*Myxococcus xanthus and Plesiocystis*) to the animal proteins points to the possibility of their origin from a HGT independent from those between actinobacteria and archaea.


MyxococcusX
PlesiocystisP
drome
Nasonia
Apis
Pediculus
Daphnia
Branchiostoma
loxl3
loxl2
loxl4
lox
loxl1
Strongylocentrotus
Oikopleura
Hydra
Ciona
Saccoglossus
PseudonocardiaD
AmycolatopsisM
Truepera_radiovictrix
SorangiumC
Haloterrigena_t.
Nocardioidaceae
Svenezuelae
Sviridiochromogenes
Scoelicolor
Slividans
Sflavogriseus
Spristinaespiralis
Sgriseus
Sscabiei
Savermetilis
Sroseosporus
Sclavuligerus
KribbellaF
StigmatellaA