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A novel laccase with urate oxidation activity from *Lysobacter* sp. T-15

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A unique urate-oxidizing enzyme was identified in a bacterium, strain T-15. Based on its phylogenetic, physiological and biochemical properties, strain T-15 was deemed to be a novel species within the genus *Lysobacter*. The enzyme expressed in *Lysobacter* sp. T-15 was composed of 592 amino acids and contained four consensus copper-binding sites, and the recombinant enzyme was, at least in this study, speculated to have three Cu ions per subunit. The primary structure of the enzyme was 33% identical to *Marinomonas mediterranea* polyphenol oxidase, but it showed no significant similarity to any known urate oxidase. With urate as the substrate, the catalytic efficiency (k_{cat}/K_m) of recombinant enzyme was $4.0 \times 10^2\ s^{-1}mM^{-1}$, and it was not inhibited by xanthine, a strong urate oxidase inhibitor. The enzyme also showed activity toward 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid), 2,6-dimethoxyphenol and bilirubin, with catalytic efficiencies of 4.9×10^2, 1.1×10^2 and $3.6 \times 10^3\ s^{-1}mM^{-1}$, respectively. We deemed the enzyme would be a member of laccase from its broad substrate specificity. However, typical laccase and other multi-copper oxidases such as bilirubin oxidase and ascorbate oxidase seldom exhibit urate oxidation activity. These results would expand the laccase substrate range to include urate.

Keywords: Laccase/*Lysobacter* sp./multi-copper oxidase/urate oxidase.

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); DMP, 2,6-dimethoxyphenol; MCO, multi-copper oxidase.

Urate oxidase (uricase, EC 1.7.3.3, Pfam PF01014) is an enzyme in the purine degradation pathway (*1*) that catalyses the oxidation of urate in the presence of O_2 and H_2O to generate 5-hydroxyisourate and H_2O_2 (*2*). Urate oxidase is commonly found in mammals, fishes and plants, and it is also seen in certain fungi, yeasts and bacteria (*3–8*) (http://www.brenda-enzymes.org/). The protein is comprised of 300–400 amino acids and exists as a homotetramer. Although prokaryotic urate oxidases share relatively little (<34%) amino acid sequence identity with the eukaryotic enzymes (*5*, *6*), two motifs are highly conserved in both (*8*). Copper and/or iron ions have been detected in urate oxidases from bacteria (*Arthrobacter pascens*, *A. globiformis*, *Bacillus fastidiosus*, *Streptomyces cyanogenus* and *Pseudomonas aeruginosa*) and eukarya (*Candida utilis* and *Sus scrofa*), though no transition-metal ions have been found in the enzymes from other sources (http://www.brenda-enzymes.org/).

Humans have lost the urate oxidase gene and therefore cannot degrade urate, which sometimes leads to gout (*3*). Urate oxidase has been commercially produced as a protein drug to reduce urate accumulation in humans (*3*, *8*), and it is also used for enzymatic determination of urate levels in human blood and urine for diagnosis of gout (*9*). However, urate oxidase is commonly inhibited by Cl^-, Br^- and NO_3^- (*4*), which prevent flexible preparation of diagnostic reagents and could cause an economic disadvantage. An enzyme resistant to these anions would therefore be preferable for diagnostic use. For this purpose, we have been seeking a Cl^--resistant urate oxidase and recently isolated a unique Cl^--, Br^-- and NO_3^--resistant urate-oxidizing enzyme from a new bacterium, designated strain T-15, which we isolated from an activated sludge. In this article, we report the primary structure, function, inhibitors and biochemical characteristics of this enzyme, and also provide polyphasic taxonomic characterization of the strain T-15.

Materials and Methods

Bacterial strain and growth conditions

Strain T-15 was used as the source of the enzyme and chromosomal DNA. The cells were aerobically grown in PE medium (*10*) at 30°C and pH 7.5. The strain was deposited in the International Patent Organism Depositary (IPOD) in Japan (FERM P-21056) as *Lysobacter* sp. T-15 based on the phylogenetic analyses described below.

Taxonomic characterization of strain T-15

Cell morphology was observed using phase-contrast light microscopy (Olympus AX80T, Olympus, Tokyo), and Gram staining was conducted using the standard method of Magee *et al.* (*11*). Oxidase and catalase activities were determined as described earlier (*12*). The temperature, NaCl and pH ranges for growth were determined using PE medium under aerobic and dark conditions. The nutritional test was performed using API 20NE and API 50CH (bioMerieux, Tokyo). Chemotaxonomic properties, including the G+C content, the major respiratory quinone and the major fatty acids, were determined as described by Zhang *et al.* (*13*).

Phylogenetic analysis of strain T-15 was performed on the basis of 16S rRNA gene sequencing. Briefly, DNA was extracted using the method of Hiraishi (*14*), and the 16S rRNA gene was amplified with primers 8F (5′-agagtttgatcctggctcag-3′) and 1492R (5′-ggttaccttgttacgactt-3′) (*15*) using an AmpliTaq Gold reagent kit (Perkin Elmer, Tokyo) according to the manufacture's protocol. The 16S rRNA gene sequence was then determined as described earlier (*13*) and compared with those in the public databases using the BLAST program. A phylogenetic tree was constructed using the neighbour-joining method (*16*) with the ARB software package (http://www.arb-home.de). Bootstrap resampling analysis (*17*) was performed with the PAUP* 4.0 package.

Enzyme assay, protein measurement and inhibition constants

The assay of urate oxidase activity is based on measurement of urate decrease. Unless otherwise specified, the following procedure was used to the assay: 40 mM potassium phosphate buffer (pH 7.0 at 25°C) and 0.115 mM urate in a total volume of 1 ml. The reaction was started by the addition of 10 µl of enzyme solution (~0.3 U/ml), after which the reaction mixture was incubated at 37°C in a cuvette, and the decrease in absorbance at 293 nm was followed spectrophotometrically ($d = 1$ cm and $\varepsilon_{293} = 12.6\,mM^{-1}cm^{-1}$). One unit of enzyme was defined as the amount catalysing the oxidation of 1 µmol of urate per min at pH 7.0 and 37°C. The activity toward 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at pH 4.6 and 37°C, bilirubin and ditaurobilirubin (an alternative serum conjugated-bilirubin) at pH 7.0 and 37°C, and ascorbate at pH 5.4 and 30°C was measured as described earlier (*18*, *19*). The activity toward 2,6-dimethoxyphenol (DMP) was measured in 100 mM citrate–NaOH buffer (pH 5.2) at 37°C by spectrophotometrically following the increase in absorbance at 480 nm ($d = 1$ cm and $\varepsilon_{480} = 13.3\,mM^{-1}cm^{-1}$). Protein was measured using a Bio-Rad protein assay kit (Bio-Rad Loboratories, Hercules, CA, USA), with bovine serum albumin serving as the standard protein. The H_2O_2 appearance was measured colorimetrically by the following reaction mixture; 40 mM potassium phosphate (pH 7.0), 1 mM urate, 0.03% 4-aminoantipyrine (4-AA), 0.02% *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS, Dojin, Kumamoto, Japan) and 10 U/ml peroxidase (Sigma-Aldrich, St Louis, MO, USA). The control experiments were done without peroxidase in the reaction mixture. K_i values were calculated from Dixon plots. The concentrations of urate used ranged from 0.05 to 1.0 mM with 0–200 mM NaCl.

Purification of the enzyme from strain T-15

All operations were carried out at room temperature. Strain T-15 cells were cultured for 38 h at 30°C, harvested by centrifugation ($8 \times 10^3 g$ for 20 min), suspended in 10 mM Tris–HCl (pH 8.0), and disrupted by ultrasonication for 15 min on ice (Cell Disruptor; Branson, CT, USA). After the cell debris was removed by centrifugation ($1.5 \times 10^4 g$ for 20 min), the supernatant was collected as the cell-free extract. The enzyme with urate oxidation activity was then partialy purifed from the supernatant using column chromatography with DEAE–sepharose Fast Flow (1 l), Phenyl Sepharose 6 Fast Flow (0.5 l) and Q Sepharose High Performance (0.2 l, GE Healthcare, Tokyo) steps, as described earlier (*18*, *20*). The Q Sepharose High Performance column chromatography fractions showing high urate oxidation activity were collected and applied to 0.1 l of Cu-chelating Sepharose (XK16, GE Healthcare) equilibrated with 10 mM sodium acetate (pH 6.5), 10% (v/v) glycerol and 0.3 M NaCl, after which the bound protein was eluted with a linear gradient (0–0.1 M) of imidazole in the same buffer. The active fractions were pooled, concentrated using a 10-kDa centrifugal filter device (Millipore, Bedford, MA, USA), and desalted by passage through a Sephadex G-25 superfine column (GE Healthcare) pre-equilibrated with 10 mM potassium phosphate (pH 7.0).

Analyses of N-terminal and inner amino acid sequences

The separated polypeptides in an SDS–PAGE gel were electroblotted onto a polyvinylidene difluoride membrane using a semi-dry blotting apparatus. The bands were then clipped from the membrane and subjected to N-terminal amino acid analysis. The urate-oxidizing enzyme purified from strain T-15 was denatured using 1 M urea, after which a reverse-phase Capcell Pak C18 HPLC column (Shiseido, Tokyo) was used to separate the enzyme solution into two fractions. The mobile phase was comprised of a 50-min linear gradient of 10–70% acetonitrile containing 0.1% trifluoroacetic acid. The flow rate was 0.5 ml/min, and the absorbance of the eluate was monitored at 215 nm. The separated polypeptides were digested with Lys-C or Asp-N endopeptidase (Roche Diagnostics, Manheim) for 17 h at 37°C, after which they were purified by HPLC, and the amino acid sequences were analysed by automated Edman degradation using an Applied Biosystems gas-phase protein sequencer (Applied Biosystems, Tokyo).

DNA cloning and sequencing

Two oligonucleotide probes, gtn(g, a, t or c)gcnccnggngay(t or c)acngtnacnggngayyt and gaycarytnggnttyggnacntgggcncc, corresponding to inner regions of the enzyme (VAPGDTVTGDL and DQLGFGTWAP) were synthesized and labelled with 740 kBq of [γ-^{32}P]ATP. The chromosomal DNA from strain T-15 was digested with several restriction enzymes, after which the resultant fragments were separated by 0.8% agarose gel electrophoresis and then subjected to Southern blotting along with the probes, as described earlier (*5*). The *Sph*I fragments of genomic DNA hybridized to the probes were recoved from the gel, ligated to pUC118 (Takara Bio, Shiga, Japan), and then transformed into *Escherichia coli* DH5α (Takara Bio). The colony hybridzation was done as described earlier (*5*). Finally, the entire gene was amplified using oligonucleotide primers 5′-atccatatgcgcgtccgttcaccgttcccg-3′ (sense) and 5′-gcggaattctcagtggctgccatgccccccatg-3′ (antisense), which contain the *Nde*I and *Eco*RI restriction sites, respectively (underlined). The amplified 2-kb fragment was digested with *Nde*I and *Eco*RI and ligated with pET21a(+) vector (Novagen, Madison, WI, USA) linearized with the same restriction enzymes to generate pET21a(+)/LMCU. *E. coli* TOP 10 cells (Invitrogen, Carlsbad, CA, USA) were then transformed with pET21a(+)/LMCU and spread onto a LB agar plate containing 50 µg/ml ampicillin. Positive colonies were selected using the colony PCR method with an Insert Check Ready Blue kit (Toyobo, Osaka). A positive transformant was then cultured in liquid LB medium containing 50 µg/ml ampicillin, after the plasmid pET21a(+)/LMCU carrying the gene was isolated from the cells, and *E. coli* strain BL21(DE3) (Novagen) was transformed with the pET21a(+)/LMCU.

Expression and purification of the recombinant enzyme from strain T-15

E. coli cells harboring pET21a(+)/LMCU were cultivated at 37 or 17°C in LB medium containing 50 µg/ml ampicillin and 0.1 mM $CuSO_4$. Expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium, as instructed in the manual for pET systems. The cells were harvested by centrifugation, suspended in 10 mM Tris–HCl (pH 7.5) containing 1 mM $CuSO_4$, and then disrupted by ultrasonication on ice. The enzyme with urate oxidation activity was partialy purifed from the lysate by column chromatography with Q Sepharose Big Beads (180 ml, GE Healthcare), Phenyl Sepharose 6 Fast Flow (50 ml) and Q Sepharose High Performance (180 ml), as described earlier (*20*, *21*). The fractions containing the urate oxidation activity were collected and desalted by passage through a Sephadex G-25 Superfine column pre-equilibrated with 10 mM potassium phosphate (pH 7.0) and concentrated using 30-kDa centrifugal filter devices. The entire operation was done at room temperature.

Cu analysis

The Cu contents were analysed by inductively coupled plasma-atomic emission spectrometry (ICP-ES, PerkinElmer, Optima 5300 DV, $n = 2$) and the procedure described by Hanna *et al.* (*22*) ($n = 5$), with CotA serving as the standard copper protein. The CotA is a spore-coat laccase from *B. subtilis* that contains four copper ions per

subunit (*18*, *23*, *24*). In the Hanna *et al.* (*22*) method, the protein samples were first diluted to 2 ml in 0.1 M potassium phosphate buffer (pH 6.5) containing 0.1 M ascorbate, and then were further diluted to 5 ml with a 2,2′-biquinoline solution (0.5 mg/ml) in glacial acetic acid. After 10 min the absorbance at 546 nm were measured. Excess ascorbate was added to reduce Cu(II) to Cu(I).

Reaction product analysis

For measurement of the reaction product in the urate oxidation reaction, a 2 × 150 mm phosphocholine hydrophilic interaction chromatography column (PC HILIC, Shiseido, Tokyo) maintained at 30°C was used for separation by an HPLC system (Agilent 1100, Agilent Technologies, CA, USA) directly coupled to a ABI QSTAR Pulsar-i mass spectrometer (Applied Biosystems). The mobile phase A was 95%/5% 10 mM ammonium acetate/CH_3CN, and mobile phase B was 100% CH_3CN. The gradient program was as follows: mobile phase B was for 5 min, followed by a gradient from 92 to 10% B was for 40 min, and 10% B was 10 min. The flow rate was 0.1 ml/min and the absorbance of the eluate was monitored at 233 nm. The stoichiometric amount was measured by a 4.6 × 250 mm PC HILCI column (Shiseido). In this analysis, an isocratic elution with 80/20% CH_3CN/H_2O was used with the flow rate of 1.0 ml/min at 40°C, and the absorbance at 210 nm of the effluent was monitored. Urate oxidase from *Bacillus* sp. (Toyobo) served as the standard reaction.

Nucleotide sequence accession numbers

The enzyme gene and 16S rRNA gene sequences have been deposited in the DDBJ database under accession nos. DL242024 and AB490175, respectively.

Results

Taxonomic characterization of strain T-15

A source microorganism of new laccase showing urate oxidation activity, strain T-15, was newly isolated from an activated sludge in a wastewater treatment process in Japan. The phylogenetic and phenotypic features were investigated in order to reveal a taxonomic position of the isolated strain.

The phylogenetic analysis besed on 16S rRNA gene from strain T-15 (1471 nt) indicated that strain T-15 was related to *Lysobacter burunescens* (97% identity), *L. gummosus* (95%), *Thermomonas brevis* (95%) and *T. haemolytica* (95%), and that strain T-15 is a member of the genus *Lysobacter* within the family *Xanthomondaceae* in the class *Gammaproteobacteria*, and formed a distinct cluster with *L. brunescens* (Fig. 1).

Colonies grown on a R2A (Becton, Dickinson and Company, Tokyo) agar plate for 3 days were circular, convex, entire, glossy, yellow and 0.5–1.2 mm in diameter. The cell morphology was a Gram-negative rod (0.3–0.5 × 4.0–7.0 μm) occuring singly and in pairs. Neither spore formation nor flagella movement was detected. Strain T-15 grew at 10–40°C, with an optimum temperature of 30°C. The pH range for growth was 5.5–9.0, with an optimum of pH 6.5–7.5. Sodium chloride inhibited growth of the strain, though the strain tolerated up to 2.0% NaCl. The strain grew on solid LB and TSB (trypticase soy broth) media, but it grew much better on low nutrient media such as 1/10 LB, 1/10 TSB, PE and R2A. Strain T-15 was an aerobic, chemoheterotrophic bacterium. Catalase and cytochrome oxidase were positive. Hydrolysis of agar and indole production were not observed. The strain was positive for assimilation of glucose, arubutin, esculin, maltose, trehalose, starch, glycogen and 2-keto-gluconate, but not glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-metyl-D-xyloside, galactose, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannose, α-methyl-D-glucose, N-acetyl glucosamine, amygdalin, salicin, cellobiose, lactose, melibiose, sucrose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 5-keto-gluconate. The strain T-15 did not show denitrifying activity. The G+C content of the total DNA was 67 mol.%. The major respiratory quinone was ubiquinone-8. The major fatty acids in strain T-15 were iso-C15:0 (50%), iso-C16:0 (16%), C16:1 (10%), iso-C17:1 (5%), C16:0 (5%), anteiso-C15:0 (5%), iso-C11:0-3OH (5%) and iso- or anteiso-C11:0 (4%).

Detection and production of the urate-oxidizing enzyme from strain T-15

When the cells were cultured in PE medium (*10*), ~6.9 mU/ml urate oxidation activity was obtained

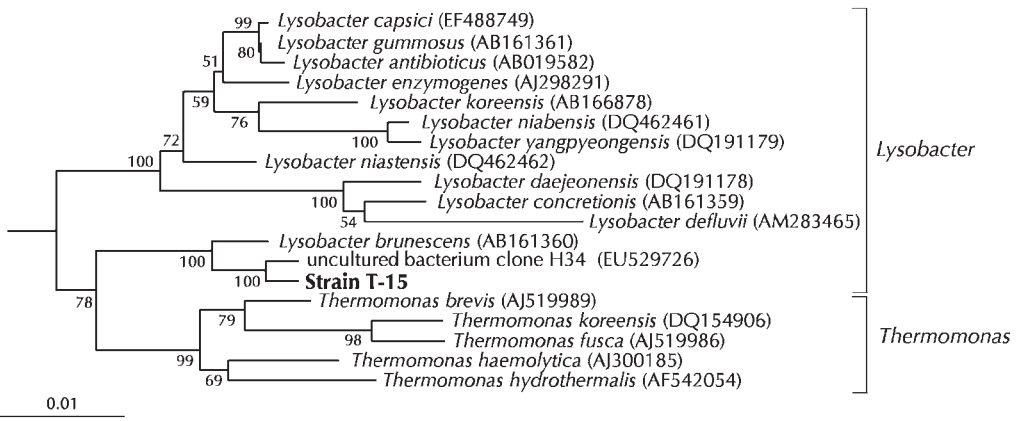

Fig. 1 Phylogenetic tree showing the relationship between strain T-15 and validly named species within the genera *Lysobacter* and *Thermomonas*. The tree is based on the 16S rRNA gene sequences and was constructed using neighbour-joining method. The scale bar indicates 0.02 substitutions per nucleotide position. Bootstrap values >50% are shown at the branch points. *Luteimonas mephitis* (accession no. AJ012228) was used as the outgroup.

after 72 h of cultivation at 30°C. Neither urate nor $CuSO_4$ addition into the medium increase the activity. The enzyme was purified over four column chromatography steps without protease inhibitors or reducing agents, and ~29 mg of the ~1,300-fold purified enzyme was obtained from 40 l of strain T-15 culture. The concentrated, purified enzyme was blue. SDS–PAGE analysis of the purified enzyme revealed the presence of two blurred polypeptides with apparent molecular masses of ~32 and 34 kDa (Fig. 2A, lane 1), whereas gel filtration analysis revealed the native enzyme to have an apparent molecular mass of ~132 kDa (Fig. 2B).

Nucleotide sequence and primary structure

Although N-terminal amino acids of the electroblotted polypeptides were not successfully sequenced, several internal sequences were able to determined as follows: DQLGFGTWAPSGRYTSING and DG AVAGRIERWRNLHAGIR in the 32-kDa fragment; DAESRRLLGTVRVAPGDTVTGDLGAYLQRQL-VEAANRWMP, DLVLGNVEEWTITSA and DYA AMKGTWK in the 34-kDa fragment. The entire gene, designated *mcu*, encoding the enzyme was cloned from a strain T-15 genomic DNA library using probes constructed on the basis of these internal sequences. The gene consisted of 1,776 nt encoding a deduced sequence of 592 amino acids with a calculated molecular mass of 64,555 Da. The sequence included the internal amino acid sequences of both the 32- and 34-kDa polypeptides, indicating that both fragments were encoded by the one open reading frame. The recombinant enzyme showed a single activity peak of ~132 kDa on gel filtration as well as the wild-type enzyme, though the specific activities of the two purified enzymes differed: 21 U/mg (wild-type) versus 42 U/mg (recombinant). The cleavage site identification of the the wild-type enzyme by analysing two chromatographically separated peptides using MS methods (Voyager-DE STR, Applied Biosystems) was unsuccessful due to inhomogeneity. A BLASTP search for the deduced amino acid sequence of the strain T-15 urate-oxidizing enzyme revealed no significant similarity to any known urate oxidases. On the other hand, it showed relatively high sequence identity with predicted multi-copper oxidases (MCOs) from the following bacterial species within the phylum *Proteobacteria*: *Hahella chejuensis* (the sequence identity was 52%), *Rhizobium etli* (51%), *Sinorhizobium medicae* WSM419 plasmid (52%) and *Nitrobacter hamburgensis* (50%). Multiple sequence alignment of strain T-15 enzyme with these MCOs revealed that the consensus copper binding sites highly conserved even in the new enzyme (Fig. 3).

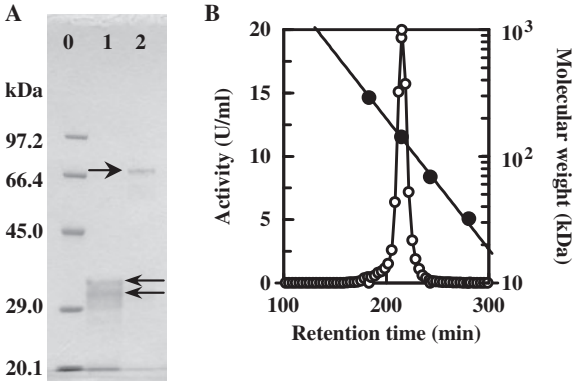

Fig. 2 Molecular weight analysis. (A) SDS–PAGE of the laccase purified from *Lysobacter* sp. T-15 (lane 1) and *Lysobacter* sp. T-15 laccase heterologously produced in *E. coli* (lane 2): lane 0, molecular mass standards; arrows, the position of the *Lysobacter* sp. T-15 laccase. (B) Molecular mass of the native laccase purified from *Lysobacter* sp. T-15 was determined by gel filtration on a Superdex 200 column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl (without the presence of reducing agents). The activities (open circles) were determined under the standard assay conditions. The molecular mass standards were (filled circles) glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (34 kDa) and cytochrome (12.4 kDa, data not shown).

Expression and purification of the recombinant enzyme

To characterize the strain T-15 enzyme with urate oxidation activity in detail, the recombinant protein was expressed in *E. coli* BL21(DE3) at low temperature of 17°C after induction with IPTG. Under these conditions, the recombinant protein was partially obtained in soluble form, and was purified 32-fold with a yield of 23% (Table I). The purified protein was an homogeneous 67-kDa polypeptide on SDS–PAGE (Fig. 2A, lane 2) and had a molecular mass of 132 kDa on gel filtration, which is consistent with that observed for the wild-type enzyme. The 10 N-terminal amino acids sequence of the protein was identical to that of the expected sequence (MTPVPDHDAS). The purified recombinant enzyme was used for the subsequent experiments.

Reaction products, substrate specificity and kinetic properties

Urate disappearance by the strain T-15 enzyme was monitored spectrophotometrically. A reaction product in the urate oxidation reaction was allantoin by LC/MS, and the reaction involved the production of H_2O_2, which was measured in the colorimetric assay. Urate degradation and allantoin formation by the *Lysobacter* sp. T-15 enzyme was in a ratio of 1 : 1 as well as that of

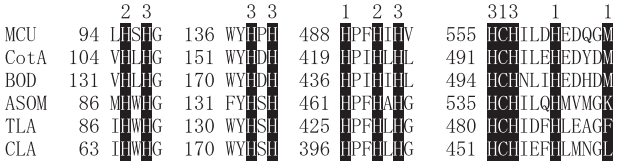

Fig. 3 Comparison of the amino acid sequences of potential copper coordination sites in MCOs: MCU, the laccase from *Lysobacter* sp. T-15; CotA, *B. subtilis* CotA (NCBI entry NP_388511); BOD, *M. verrucaria* bilirubin oxidase (NCBI entry Q12737); ASOM, *Acremonium* sp. ascorbate oxidase (EMBL AB010110); TLA, *Trametes villosa* laccase (NCBI entry AAB47735); CLA, *Coprinus cinereus* laccase (NCBI entry 1HFUA). The numbers 1, 2 and 3 indicate the potential coordination sites for types-1, 2 and 3 copper ions, respectively. The amino acid residues presumed to be involved in binding copper are depicted in black boxes.

authentic urate oxidase. Because the amino acid sequence of the enzyme contained four consensus MCO family motifs, the substrate specificity of the enzyme towards the following general MCO substrates were examined: 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), bilirubin and ascorbate. The enzyme showed activity towards ABTS, DMP and bilirubin, exhibiting typical Michaelis–Menten kinetics (Fig. 4), but showed little or no activity towards ascorbate (Table II). Using DMP as the substrate, the K_m and V_{max} values for the strain T-15 enzyme were 4.7 mM and 5.0×10^2 μmol/min/mg ($k_{cat} = 5.4 \times 10^2\ s^{-1}$), respectively. The highest V_{max} value was observed with ABTS ($k_{cat} = 1.3 \times 10^3\ s^{-1}$), while bilirubin was the most preferred substrate with the highest V_{max}/K_m value ($k_{cat}/K_m = 3.6 \times 10^3\ s^{-1}mM^{-1}$). No H_2O_2 generation was observed in the laccase-like reaction catalysed by the enzyme with typical laccase substrates. The enzyme showed similar V_{max}/K_m values for urate and ABTS ($k_{cat}/K_m = 4.0 \times 10^2$ and $4.9 \times 10^2\ s^{-1}mM^{-1}$, respectively). With urate as the substrate, the V_{max}/K_m ratio of *Lysobacter* sp. T-15 enzyme was similar to that of the *A. globiformis* urate oxidase, and the K_m was 36 and 20 times greater than those of cowpea and soybean urate oxidase, respectively (10 and 18 μM, respectively) (*25*). Examination of V_{max}/K_m values for bilirubin and urate reveals that urate is almost 10-fold poorer as a substrate. With ABTS as the substrate, the V_{max}/K_m ratio was twice that of CotA, 14% that of bilirubin oxidase and <0.1% that of laccase (*26*). For bilirubin, the V_{max}/K_m ratio was similar to that of CotA and 26 times higher than that obtained with *M. verrucaria* bilirubin oxidase (*27*). Xanthine, a strong inhibitor of known urate oxidases, had no effect on the urate oxidation activity of the strain T-15 enzyme (Table III). In addition, Cl^-, Br^- and NO_3^-, which reportedly inhibit soybean root nodule urate oxidase (*4*), and inhibited urate oxidases from *A. globiformis*, *Bacillus* sp. and *Candida* sp. in the present (Table III), had substantially less effect on strain T-15 enzyme. The K_i for the inhibitory effect of NaCl on *A. globiformis* urate oxidase was 5.7 mM (non-competitive). By contrast, the K_i for the effect on urate oxidation activity of the strain T-15 enzyme was 200 mM (competitive). The enzyme also catalysed the condensation reaction of 4-AA and DAOS (1.9 μmol/min/mg).

Table I. Purification of *Lysobacter* sp. T-15 laccase heterologously produced in *E. coli*.

Step	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)
Cell free ext.	9,000	7,000	1.3	100
Q sep. BB	4,800	390	12	53
Phenyl sep. ff	3,500	120	29	39
Q sep. HP	2,100	50	42	23

The cell extract was prepared from 1.61 of culture.

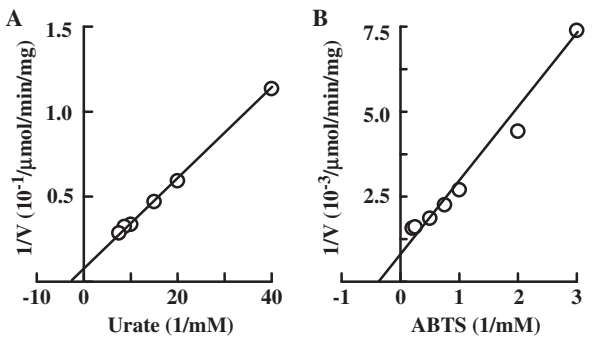

Fig. 4 Double-reciprocal plots of activity versus (A) urate and (B) ABTS.

Other biochemical properties

Other biochemical properties of the strain T-15 urate-oxidizing enzyme are summarized in Table IV. The urate degradation activity of the enzyme increased up to a temperature of ~58°C; above this temperature, activity declined probably due to decomposition of the enzyme. The Arrhenius plot showed two-phase linearity with a transition point ~40°C, which is generally interpreted as being due to a change in conformational flexibility (Fig. 5A) (*28*). When urate or bilirubin was used as the substrate, little or no activity was observed in the pH ranges of 4.2–5.0 and 9.0–10.0. With ABTS or ditaurobilirubin as a substrate, the activity was only about half of maximum, even at a pH range of 3.5–4.2, and <10% of maximal activity was observed in the range of pH 9.0–10.0. The enzyme was fully stable for up to 15 min at a temperature of 40°C, lost 60% of its activity at 60°C, and was completely inactivated by the incubation for 15 min at 80°C. The enzyme exibited a pronounced absorption peak at ~610 nm and a tailing around 310 nm, but the latter absorption was less apparent, mainly because of overlapping with the strong absorption of the aromatic protein residues (Fig. 5B). The Cu content of the enzyme was estimated to be 3.2 ± 0.2 par molecule by ICP-ES. Other than Cu, no significant level of metals, e.g. Zn, Mn, Fe, Co, Ca, Mg and Al, were detected. The Cu content was also estimated according to the procedure described by Hanna *et al.* (*22*). The similar result, 2.6 ± 0.5 Cu/subunit, was obtaind in the additional estimation, suggesting that the enzyme contained three Cu ions/subunit.

Discussion

The new enzyme that exhibits urate oxidation activity in addition to the laccase activity was found in a bacterium, designated strain T-15 newly isolated from activated sludge in Japan. Phylogenetic analysis based on the 16S rRNA gene sequence showed that strain T-15 is affiliated with the genus *Lysobacter* within the family *Xanthomonadaceae* in the class *Gammaproteobacteria* and, with 97.5% sequence identity, is most closely related to *L. brunescens* (Fig. 1). The physiological and biochemical features of strain T-15, including its yellow pigmentation, chemotaxonomic traits and so on, were also similar with *L. brunescens*. However, the strain also possessed phenotypic characteristics that obviously differed

Table II. Comparison of the kinetic properties of *Lysobacter* sp. T-15 laccase heterologously produced in *E. coli* with those of other MCOs and urate oxidase.

	Lysobacter sp. T-15 laccase	CotA[a]	Bilirubin oxidase	Ascorbate oxidase[b]	Laccase[c]	Urate oxidase[d]
Urate						
K_m (mM)	0.36					0.07
V_{max} (μmol/min/mg)	133	±	±	nd	±	24
V_{max}/K_m	369					343
ABTS						
K_m (mM)	2.8	0.106[e]	0.5[f]	100	0.0128[g]	nd
V_{max} (μmol/min/mg)	1,250	22[e]	2,500[f]	600	8,125.4[g]	
V_{max}/K_m	446	208	5,000	6	634,797	
Bilirubin						
K_m (mM)	0.015	0.008[h]	0.19[i]			
V_{max} (μmol/min/mg)	49	28[h]	24[j]	±	±	nd
V_{max}/K_m	3,267	3,500	126			
Ascorbate						
K_m (mM)				0.3		
V_{max} (μmol/min/mg)	±	±	±	1,800	±	nd
V_{max}/K_m				6,000		

Michaelis constants were determined from the initial rates. The concentrations of substrates used were: 0.05–1 mM urate; 0.2–5, 0.1–10 and 1–25 mM ABTS for *Lysobacter* sp. T-15 laccase, bilirubin oxidase and ascorbate oxidase, respectively; 0.002–0.068 mM bilirubin; and 0.05–1 mM ascorbate. [a]Spore-coat laccase from *Bacillus subtilis*, also known as bilirubin oxidase (Asahi Kasei Pharma, Tokyo), [b]from *Acremonium* sp. (Asahi Kasei Pharma), [c]from *Trametes versicolor* (Sigma-Aldrich), [d]from *Arthrobacter globiformis* (Asahi Kasei Pharma) (*5*), [e]from Martins *et al.* (*24*), [f]from *Trachyderma tsunodae* K-2593 (Takara Bio, Shiga) (*29*), [g]from Han *et al.* (*26*), [h]from Sakasegawa *et al.* (*18*), [i]from Tanaka and Murao (*27*) and [j]from Shimizu *et al.* (*36*). ±, less than 0.1 μmol/min/mg. nd, not detected.

Table III. Effects of various urate oxidase inhibitors on urate oxidation activities.

	Lysobacter sp. T-15 laccase	Urate oxidase[a] *Arthrobacter globiformis*	Urate oxidase[b] *Bacillus* sp.	Urate oxidase[b] *Candida* sp.
1 mM xanthine	97	39	22	35
5 mM xanthine	101	16	0	10
10 mM NaCl	98	71	88	57
10 mM KCl	97	72	90	59
10 mM NaBr	104	74	90	60
50 mM $NaNO_3$	105	63	85	50
10 mM Na_2SO_4	101	97	96	98
10 mM CH_3COONa	99	97	100	99

Data are percentages of maximum activity under the standard assay conditions. Activities were measured in the presence of the indicated compounds. [a]Asahi Kasei Pharma (*5*), [b]Toyobo, Osaka.

Table IV. Biochemical properties of the *Lysobacter* sp. T-15 laccase heterologously produced in *E. coli*.

Parameter	Value
Molecular mass	
Native enzyme (kDa)	132
Calculated (Da)	64,555
pI[a]	5.2
pH optima[b]	
Urate	6.6–6.9
ABTS	4.8–5.2
Bilirubin	6.6–7.0
Ditaurobilirubin	4.8–5.2
Thermostability[c]	<40°C

[a]Isoelectric focusing was done for 40 h at 25°C with carrier ampholytes giving a pH gradient of 3.5–10 on a 110 ml electrofocusing column. [b]The following overlapping buffers were used to cover the entire pH range tested under the standard assay conditions: acetate–NaOH (pH 3.5–5.5), citrate–NaOH (pH 4.5–6.0), potassium phosphate (pH 6.0–7.5), and Tris–HCl (pH 7.5–10.0). [c]To assess thermostability, aliquots of enzyme in 40 mM potassium phosphate (pH 7.0) (2.5 U/ml) were incubated in sealed tubes for 15 min at temperatures ranging from 0 to 80°C. After incubation, all tubes were rapidly cooled in an ice bath and analysed for activity.

from *L. brunescens*: (i) strain T-15 does not grow at 45–50°C, whereas *L. brunescens* does; (ii) cells of strain T-15 are 4–7 μm long, while those of *L. brunescens* are 7–70 μm long; (iii) strain T-15 forms circular colonies, whereas *L. brunescens* forms thin-spreading colonies, indicating gliding motility; (iv) strain T-15 shows neither indole production nor agar-degradation, but *L. brunescens* does so; (v) strain T-15 can utilize maltose, glycogen and D-glucose as substrates, but *L. brunescens* cannot. On the basis of polyphasic taxonomic analyses, strain T-15 can be classified a novel species within the genus *Lysobacter*, i.e. *Lysobacter* sp. T-15.

The enzyme newly found in *Lysobacter* strain T-15 was considered as one of MCOs for the following reasons: (i) the presence of conserved copper binding domains in the gene sequence (Fig. 3); (ii) the enzymatic activities observed using typical MCO substrates; (iii) an absorption spectrum showing the absorption of the blue type-1 copper (~610 nm, Fig. 5B); (iv) the actual presence of Cu ions. In particular, this enzyme would be appropriately a member of laccase from its substrate specificity, because it is known that the MCO family enzymes, especially laccase, oxidize a wide variety of substrates. The enzyme also catalysed 4-AA and DAOS coupling reaction typically found in laccases. To date, however, no laccase showing urate oxidation activity has been reported. The *Lysobacter* sp. T-15 urate-oxidizing enzyme is, therefore, the first and only laccase able to oxidize urate. The comparison of substrate specificities with commercially available MCOs and *A. globiformis* urate oxidase demonstrated that the *Lysobacter* sp. T-15 enzyme has distinct enzymatic feature different from either other MCOs or a

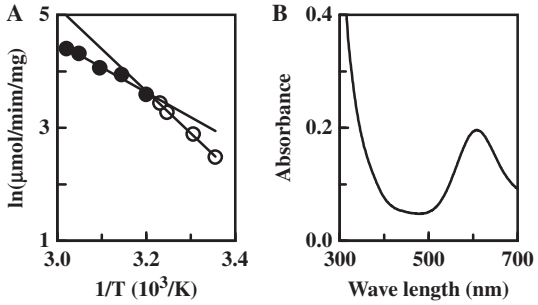

Fig. 5 Arrhenius plot and absorption spectrum. (A) Arrhenius plot of the urate degradation activity of the laccase heterologously produced in *E. coli*. From the slopes of the linear parts in the Arrhenius plot, an activation energy of 62 kJ/mol was calculated for the temperature range between 25 and 36.5°C (open circles) and of 37 kJ/mol for the temperature range between 39.5 and 58°C (filled circles). (B) Absorption spectra of the recombinant *Lysobacter* sp. T-15 laccase. The solution was comprised of 18 mg/ml enzyme in 10 mM potassium phosphate (pH 7.0).

common urate oxidase (Table II). The *Lysobacter* sp. T-15 enzyme could oxidize usual substrates of MCOs, i.e. ABTS and bilirubin in addition of urate. Other MCOs, CotA, bilirubin oxidase from *Trachyderma tsunodae* (*29*), ascorbate oxidase from *Acremonium* sp., and laccase from *Trametes versicolor* (*26*), all showed no significant ability to oxidize urate, and a common urate oxidase from *A. globiformis* (*5*) was not able to oxidize ABTS, bilirubin or ascorbate. These findings indicate that the *Lysobacter* sp. T-15 urate-oxidizing enzyme is a unique laccase that utilize urate in addition to the standard laccase substrates and is clearly distinct from typical urate oxidases.

The reaction products in the urate oxidation reaction by *Lysobacter* sp. T-15 enzyme were possibly 5--hydroxyisourate and H_2O_2 like authentic uricase, and produced 5-hydroxyisourat spontaneously decomposed to allantoin and CO_2 (*2*). Urate oxidation activity implies a two-electron reaction between urate and O_2 to produce H_2O_2 and supposedly 5-hydroxyisourate. In all known MCOs, O_2 reacts with the reduced trinuclear copper cluster of the enzyme to produce two water molecules in a four-electron process. The electrons needed to drive this reaction are supplied by the sequential reduction of the type-1 copper by four substrate molecules, which donate one electron at a time (*30–32*). Thus, this mechanism would be inconsistent with the two-electron reaction needed to oxidize urate. Release of peroxide has never been observed during enzymatic turnover of any wild-type MCO. Even in 'type-1 depleted' MCOs where the O_2 reduction is halted at the level of peroxide (*32*), the peroxide is bound stably to the trinuclear copper cluster and does not dissociate from the enzyme. It may be reasonable to propose that urate is oxidized by a single electron enzymatically because laccases catalyse the transfer of a single electron at a time, and the formation of H_2O_2 arises from further non-enzymatic reactions from superoxide.

There is another inconsistency between the observation of four consensus Cu-binding sites and the possibility of three Cu per subunit in this study, because most laccases contain a trinuclear copper cluster. Although the enzyme had four consensus Cu-binding sites, the estimation by ICP-ES and quantitative analysis by Hanna *et al.* (*22*) suggested that the enzyme contained three Cu.

The *Lysobacter* sp. T-15 laccase showed a strong resistance to xanthine, a potent urate oxidase inhibitor. Urate oxidase is an enzyme located in the purine degradation pathway, and its activity is strictly regulated by xanthine (*33*), a substrate of xanthine dehydrogenase (EC 1.17.1.4) in the same pathway. The lack of regulation by xanthine suggests the enzyme has no relevance to the purine catabolic pathway, although a metabolic role of the *Lysobacter* sp. T-15 enzyme is still unknown. This enzyme could be adapted to a variety of diagnostic reagent compositions because it was thus insensitive to xanthine and certain anions.

A multiple sequence alignment of the *Lysobacter* sp. T-15 laccase with several previously characterized MCOs is shown in Fig. 3. It has been proposed that the differences in substrate specificity among MCOs reflect the heterogeneity of their amino acid sequences within the consensus domains (*27*). X-ray crystallographic analysis of the structure of *Copinus cinerius* laccase showed that the type-1 copper center of the enzyme contains H396, C452 and H457 as the copper ligands and a non-ligating L462 at a position axial to the ligand (*34*). Kumar *et al.* (*35*) reported that most fungal laccases carry L or F at the axial position, 10 residues downstream from C452 (Fig. 3), though several other MCOs, including the *Lysobacter* sp. T-15 laccase, feature M at this position. Within the consensus domain, particular amino acids conserved between bilirubin oxidases (CotA) from *B. subtilis* and *M. verrucaria* are L106, D154, I421, L425 and D501 (Fig. 3, CotA numbering) (*18*, *23*, *24*, *36*). However, in two bilirubin oxidases and the *Lysobacter* sp. T-15 laccase, these amino acids are not conserved.

The recombinant protein had a molecular mass of 132 kDa and a subunit size of 64,555 Da (Table IV); the enzyme thus resembled typical MCO family enzymes in respect of subunit size and homodimeric structure (*37*). Urate oxidase is generally a homotetramer with a subunit size of ~33 to 44 kDa, and the characteristics clearly differed from the *Lysobacter* sp. T-15 enzyme.

The pI of this enzyme was significantly lower than that of CotA from *B. subtilis* (pH 7.7) (*23*, *24*) or laccase from *T. thermophilus* HB27 (>7.18) (*38*). In the present study, the pI of *Lysobacter* sp. T-15 laccase was calculated from the sequence to be 6.2, but was experimentally determined to be 5.2. Discrepancies between the pI calculated from the sequence and that seen in practice have been reported for laccases from *B. subtilis* and *T. thermophilus* HB27 (*24*, *38*).

While the optimum pH for bilirubin oxidation by *Lysobacter* sp. T-15 enzyme was near neutral, those for ABTS and ditaurobilirubin were about pH 5 (Table IV). It was reported that Bilirubin oxidase and laccase also oxidized ABTS and ditaurobilirubin at acidic pH (*37*).

The *Lysobacter* sp. T-15 enzyme was thermosensitive (Table IV), unlike many of the other bacterial laccases were thermostable (*24*, *38*, *39*, *40*). The *Lysobacter* sp. T-15 laccase lost 85% of its laccase activity after incubation for 10 min at 70°C. Enguita *et al*. (*41*) inferred in their article that a higher proline content of the laccase would be connected with its thermostability. However, the *Lysobacter* sp. T-15 laccase contained 58 proline residues (8.8% of the total number of residues sequences), and the number was almost identical to that in the thermostable laccase (8.9%). This finding strongly suggests that thermostability of laccases cannot be due only to its proline content but also to other structural features.

Conflict of interest
None declared.

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