A soil-borne Mn(II)-oxidizing bacterium of *Providencia* sp. exploits a strategy of superoxide production coupled to hydrogen peroxide consumption to generate Mn oxides

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Abstract
Bacterial non-enzymatic Mn(II) oxidation involving reactive oxygen species (ROS) (i.e., indirect oxidation), initially discovered from a marine alpha-proteobacterium, is believed to be of importance in controlling biogeochemical cycles. For soil-borne bacteria, however, evidence of indirect Mn(II) oxidation remains unclear. In this study, the indirect Mn(II) oxidation was evidenced in a soil-borne bacterium, *Providencia* sp. LLRDA6. First, with and without 50 mM of Mn(II) exposure for LLRDA6, 300 differentially expressed genes were found to be linked to Mn(II) exposure via transcriptome sequencing. Among them, an operon, responsible for phenylacetic acid catabolism, was sharply upregulated in transcription, drawing us a special attention, since its transcriptional upregulation has recently shown to be important for withstanding ROS. Next, a fluorometric probe, 2′,7′-Dichlorofluorescin diacetate (DCFDA), was used to qualitatively detect ROS from cells, showing a distinct increase in fluorescence intensities of ROS during Mn(II) exposure. Furthermore, concentrations of superoxide and hydrogen peroxide from cells were detected, respectively, with and without Mn(II) exposure, exhibiting that when Mn(II) oxidation occurred, superoxide concentration significantly increased but hydrogen peroxide concentration significantly decreased. Particularly, superoxide produced by LLRDA6 was proven to be the oxidant for Mn(II) in the formation of Mn oxides. Finally, we predicted links between phenylacetic acid metabolism pathway and ROS during Mn(II) exposure, proposing that the excessive ROS, generated in response to Mn(II) exposure, transcriptionally activate phenylacetic acid catabolism presumably by increasing concentrations of highly reactive oxepins.

Keywords *Providencia* sp. · Mn(II) oxidation · Reactive oxygen species · Phenylacetic acid catabolism

Introduction
The oxidation of soluble Mn(II) to insoluble Mn(III/IV) minerals plays a critical role in global biogeochemical cycles, greatly affecting the fate and transport of trace elements, the cycling of carbon-based nutrients, and the activity of microbial metabolisms (Learman et al. 2011a). Over 30 different types of Mn (oxyhydr) oxides (hereon Mn oxides), with layered or tunnel structures, occur in a wide variety of environmental settings from oceans to soils (Hens et al. 2016). These ubiquitous minerals exhibit high binding affinity to metal ions because of their large surface-to-volume porous structure, and are often used as one of the strongest metal scavengers in the environment (Butterfield et al. 2016). Moreover, due to the presence of unstable, highly reactive Mn(III) in these minerals, Mn oxides are powerful oxidants, capable of degrading a number of recalcitrant organic matters (Learman et al. 2011a). Apart from strong sorptive and oxidative capacity, Mn oxides are often involved in microbial metabolisms. In the absence of oxygen, Mn oxides (e.g., MnO2) can be terminal electron acceptors, supporting the respiration and growth of certain anaerobic Mn-reductive bacteria (Henkel et al. 2019; Lin et al. 2015).
Despite being thermodynamically favorable for the oxidation of Mn(II) by O$_2$ in the environment, most naturally occurring Mn oxides are likely attributed to bacterial Mn(II) oxidation, because Mn(II)-oxidizing bacteria play a dominant role in accelerating the rate of Mn biomineralization in nature, which is several orders of magnitude faster than abiotic pathways (Tebo et al. 2004; Morgan 2005; Butterfield et al. 2016). In general, two protein families perform direct Mn(II) oxidation coupled to O$_2$ reduction: the multicopper oxidases (MCOs), and the peroxidase cyclooxygenases (Lingappa et al. 2019).

Previous studies have shown that MCOs and their gene clusters have been identified in a diverse array of bacteria, such as Bacillus sp. PL-12 (mnx) (Dick et al. 2008a), Leptonothrix discophora SS-1 (mof) (Corstjens et al. 1997), Pseudomonas putida GB-1 (mcoA and mnxG) (Geszvain et al. 2013), Bacillus sp. SG-1 (mnx) (van Waasbergen et al. 1996; Francis et al. 2002), and Pedomicrobium sp. ACM 3607 (mox) (Larsen et al. 1999; Ridge et al. 2007). Usually, these MCOs share low homology except for their copper binding motifs (Ridge et al. 2007), which contain a unique Cu(II) center (type I Cu) and a trinuclear Cu cluster composed of one type II and two type III Cu atoms (Kataoka et al. 2007). Although a variety of gene clusters of MCOs have been identified, isolation of the responsible enzymes is still a challenge (Butterfield et al. 2013; Soldatova et al. 2017a). Among these enzymes, MnxG, has now been successfully isolated from Bacillus sp. PL-12, together with accessory proteins MnxE and MnxF as a multimERIC complex (known as Mnx), by expressing four contiguous genes as part of an operon in E. coli (Butterfield et al. 2013). Recently, the mechanism of Mn(II) oxidation by protein Mnx has been shown to exploit the polynuclear chemistry of Mn, in the oxidation states II, III, IV, to nucleate MnO$_2$ nanoparticles (Soldatova et al. 2017a, 2017b, 2019).

In addition to MCOs, another class of bacterial proteins responsible for direct Mn(II) oxidation is described as peroxidase cyclooxygenase. Strictly speaking, these proteins show a certain degree of sequence homology to animal heme peroxidases (AHPs), which belong to peroxidase cyclooxygenase superfamilies of peroxidases (Anderson et al. 2009; Nakama et al. 2014). Like MCOs, they are believed to be outer membrane proteins, with no direct involvement in energy conservation (Lingappa et al. 2019). In addition, these enzymes adopt a way of single electron transfer to form an intermediate of Mn(III), which is further oxidized to Mn(IV) oxides via disproportionation, or by another MCOs-type enzyme (Schlosser and Höfer 2002; Nakama et al. 2014). Now, these AHPs-like enzymes, consisted of animal heme peroxidase domains and hemolysin-type Ca(II) binding domains, have been named Mn(II)-oxidizing peroxidases (MopA), as found in Aurantimonas manganoxydans SI85-9A1 (Anderson et al. 2009; Dick et al. 2008b), Erythrobacter sp. SD-21 (Anderson et al. 2009; Nakama et al. 2014) and Pseudomonas putida GB-1 (Geszvain et al. 2016). In A. manganoxydans SI85-9A1 and P. putida GB-1, MopA has two animal heme peroxidase domains and two Ca(II) binding domains. Comparatively, in Erythrobacter sp. SD-21, MopA has only one animal heme peroxidase domain and one Ca(II) binding domain (Anderson et al. 2009; Nakama et al. 2014). Therefore, mechanisms of direct Mn(II) oxidation by MopA may be different in these bacterial species. For instance, hydrogen peroxide stimulated the activity of MopA identified in A. manganoxydans SI85-9A1, but had no positive effect on MopA in Erythrobacter sp. SD-21, which required NAD$^+$ rather than hydrogen peroxide for Mn(II) oxidation (Anderson et al. 2009; Nakama et al. 2014; Johnson and Tebo 2008; Medina et al. 2018).

Apart from the two above-mentioned ways of direct Mn(II) oxidation by bacteria, nowadays, a third pathway of Mn(II) oxidation by bacteria, i.e., indirect oxidation, attracts more and more attention, after widespread discovery of production of extracellular superoxide by heterotrophic bacteria (Diaz et al. 2013). An example of indirect Mn(II) oxidation is well described in Roseobacter sp. AzwK-3b, a representative within the Roseobacter clade that comprises the ca. 20% of marine bacterial communities (Learman et al. 2011b; Learman and Hansel 2014; Andeer et al. 2015; Hansel et al. 2019). In this case, superoxide (O$_2^-$), one of reactive oxygen species (ROS), probably generated by extracellular AHPs-like enzymes, dehydrogenases and NADH-oxidoreductases, plays a central role in Mn(II) oxidation. However, a high level of superoxide produced by bacteria cannot ensure their generation of Mn oxides (Diaz et al. 2013; Learman and Hansel 2014). Theoretically, the removal of hydrogen peroxide is necessary to pull the equilibrium (the reaction Eq. 1) towards accumulation of Mn(III), thus minimizing the back reaction of Mn(III) reduction (Lingappa et al. 2019; Andeer et al. 2015). Therefore, both superoxide production and hydrogen peroxide consumption are required for ROS-dependent generation of Mn oxides (Andeer et al. 2015):

\[
\text{Mn(II)} + \text{O}_2^- + 2\text{H}^+ \leftrightarrow \text{MnO}_2^2 + 2\text{H}^+ \leftrightarrow \text{Mn(III)} + \text{H}_2\text{O}_2
\]  

(1)

To date, it is generally accepted that the mechanism of bacterial direct-Mn(II) oxidation is better understood than that of indirect-Mn(II) oxidation. Evidence for bacterial indirect Mn(II) oxidation is mostly obtained from the marine bacterium Roseobacter sp. AzwK-3b. Thus, discovery of new bacterial species of indirect Mn(II) oxidation from different environments will benefit to deepen the understanding of the link between Mn cycling, ROS and health of microbial populations (Andeer et al. 2015; Hansel et al. 2019), because indirect Mn(II) oxidation may involve numerous bacteria from oceans to soils, significantly contributing to global biogeochemical cycles (Diaz et al. 2013). Recently, we identified a Mn(II)-oxidizing bacterium from soils, Providencia sp. LLDRA6, a
powerful scavenger for heavy metals from soils because of its high production of Mn oxides (Li et al. 2020). Here, we explored the mechanism of Mn oxides generation byProvidencia sp. LLDRA6, using a combination of genome sequencing, transcriptomic analyses and chemical activity assays. Collectively, these data demonstrated that this bacterium was an ROS-dependent producer of Mn oxides.

Materials and methods

Strain and incubation conditions

Providencia sp. LLDRA6 is a Gram-negative bacterium (China Center for Type Culture Collection: M2018876; Korean Collection for Type Cultures: KCTC 92091), originally isolated from soil samples near a smelting factory (113°09′11.48″ E and 27°87′07.41″ N) at Zhuzhou, Hunan Province, China, exhibiting high tolerance and strong oxidation ability for Mn(II) (Li et al. 2020). The strain LLDRA6 was finally isolated from soil samples near a smelting factory (113°09′11.48″ E and 27°87′07.41″ N) at Zhuzhou, Hunan Province, China, exhibiting high tolerance and strong oxidation ability for Mn(II) (Li et al. 2020). The strain LLDRA6 was incubated in the dark using the sterile Luria–Bertani (LB) liquid medium (5 g L⁻¹ of yeast extract powder, 10 g L⁻¹ of peptone, 5 g L⁻¹ of sodium chloride, and pH = 7) in a rotary shaker with 180 rpm at 35 °C. The incubation conditions of darkness, 180 rpm and 35 °C for cell growth were maintained in all experiments unless otherwise noted. The chemical reagents prepared for LB media were purchased from Sinopharm Chemical Reagent Co., Ltd, China.

Genome summary of Providencia sp. LLDRA6

Data of genomic sequencing for LLDRA6 by PacBio RS II and Illumina HiSeq 4000 platforms at the Beijing Genomics Institute (BGI, Shenzhen, China) are deposited at the Genbank with accession number of CP067099. The clean reads of genome sequencing are deposited in the NCBI Sequence Read Archive under BioProject PRJNA724814. The genome sequence of 4.34 Mb was obtained with no gaps by assembling 1131 Mb Illumina data (260 coverage) with 1497 Mb PacBio data (344 coverage), consisting of a circular chromosome with a G+C content of 40.18%. A total of 3956 genes were found, of which 3818 genes (96.51%) were functionally annotated via cluster of orthologous groups of proteins (COG). The principal features for the genome of strain LLDRA6 are shown in Fig. S1 and Table S1.

Determination of the optimum time of cell incubation and Mn(II) exposure for Mn(III) accumulation by strain LLDRA6 via trapping assays of Mn(III)-intermediate by the ligand pyrophosphate

The optimum time parameters of incubation and Mn(II) exposure for cells to accumulate Mn(III) were determined via trapping assays of Mn(III)-intermediate by the ligand pyrophosphate (PP) as previously described (Thi et al. 2018). Briefly, a single colony of strain LLDRA6 obtained from the LB agar plate was incubated for 12 h in the LB liquid medium. Then, 100 μL of cell suspension (ca. 1.0–1.5 × 10⁷ CFU/mL) were inoculated into a fresh 50 mL of LB liquid medium (at a volume ratio of 0.2%), incubating for 4 h, 8 h, 12 h, and 24 h, respectively. Subsequently, stock solutions of 1 M of MnCl₂ and 200 mM of Na₄P₂O₇ (sodium pyrophosphate, S108847, Aladdin) were simultaneously added into cell suspensions, both reaching a final concentration of 0.5 mM, with cells being constantly incubated for different times ranging from 0 to 36 h, i.e., time for cells to be exposed with Mn(II). After that, the adsorption spectra of Mn(III)–PP complexes at different Mn(II) exposure times were monitored using an ultraviolet–visible (UV–Vis) spectrophotometer (TU-1810, Pgeneral, China) in a range of 200–600 nm.

Experimental design for transcriptome sequencing of strain LLDRA6 with and without Mn(II) exposure

To obtain information on differentially expressed genes (DEGs) between the short-term and the long-term exposure of Mn(II), four types of cDNA libraries for transcriptome sequencing were constructed from strain LLDRA6 according to the methods as described by Zhang et al. (2018), including LLDRA6 with a short-term exposure of 50 mM of Mn(II), LLDRA6 with a long-term exposure of 50 mM of Mn(II), and two controls of LLDRA6 with no exposure of Mn(II) in the corresponding periods, which were denoted as the Mn(II)-short group, the Mn(II)-long group, the CK-short group, and the CK-long group, respectively. The flowchart of experimental design for transcriptome sequencing is shown in Fig. S2.

Specifically, according to results of the above-mentioned Mn(III) trapping assays (“Determination of the optimum time of cell incubation and Mn(II) exposure for Mn(III) accumulation by strain LLDRA6 via trapping assays of Mn(III)-intermediate by the ligand pyrophosphate”), the optimum incubation time when cells are the most active for Mn(III) production (denoted as a h, which could be any one among 4, 8, 12, and 24 h), and the optimum time for cells to accumulate Mn(III) (denoted as b h, which could be any one in the range of 0–36 h), were determined as the time parameters for cell incubation and the long-term Mn(II) exposure prior to transcriptome sequencing, respectively. For short-term Mn(II) exposure, its time parameter was set as 0.5 h, considering that on the one hand, it is unlikely to generate Mn oxides by Mn(II)-oxidizing bacterium in such a short time. On the other hand, such a short time of Mn(II) exposure would result in a wide range of transcriptional alteration in cells due to stress responses, of which the majority...
may not really attach to Mn(II) oxidation and thus could be served as a reference when comparing with transcriptional data obtained from the long-term Mn(II) exposure. In addition, the concentration of 50 mM used in Mn(II) exposure was determined according to the results of our previous study, in which strain LLDDRA6 showed the best yield of Mn oxides at 50 mM of Mn(II) (Li et al. 2020).

In detail, a single colony from the LB agar plate was incubated for 12 h in the LB liquid medium. Subsequently, cell suspension (ca. 1.0 ~ 1.5 × 10^7 CFU/mL) were inoculated into a fresh LB liquid medium at a volume ratio of 0.2%, incubating for a h in advance. After that, the cells were immediately exposed to 50 mM of Mn(II) by adding the stock solution of MnCl₂ to the cell suspension. After 0.5 h (short-term exposure) or b h (long-term exposure) of further incubation, the cells of Mn(II)-short group or Mn(II)-long group were harvested by centrifugation for subsequent transcriptome sequencing. Meanwhile, cells incubated with no exposure of Mn(II) in the corresponding periods (i.e., a + 0.5 h and a + b h, respectively) were regarded as controls (i.e., CK-short group and CK-long group, respectively). Each treatment group consisted of three biological replicates.

**Data analysis of transcriptome sequencing**

Transcriptome sequencing data of twelve cell samples obtained using an Illumina HiSeq 4000 platform at the Beijing Genomics Institute (BGI, Shenzhen, China), are deposited at Sequence Read Archive in NCBI (BioProject PRJNA690198).

After RNA sequencing (RNA-seq), clean reads of twelve cell samples were mapped to the reference genome (Fig. S1) by the STAR software (Dobin et al. 2013), and then transcript quantification was carried out by the RSEM software (Li and Dewey 2011). TPM (Transcripts Per Kilobase Million) analysis was used to calculate gene expression levels, and then the edgeR was used to investigate DEGs between different groups as instructed in “Experimental design for transcriptome sequencing of strain LLDDRA6 with and without Mn(II) exposure” (three independent biological replicates for each group), using a TRIZol™ Max™ Bacterial RNA Isolation Kit (16096020, Invitrogen) according to the manufacturer’s instructions. Subsequently, the first-strand cDNA of RNA samples was synthesized by reverse transcription using a SuperScript IV First-Strand Synthesis System Kit (18091050, Invitrogen) according to the manufacturer’s instructions. For each PCR, 1 μL of template cDNA was mixed with 12.5 μL of 2 × SYBR Green PCR Master Mix (Roche, Basel, Switzerland) and 5 pmol of each forward and reverse primer in a final reaction volume of 25 μL. Amplification program was performed as following reaction conditions: 15 s at 95 °C, followed by 15 s at a temperature 5 °C below the primer’s true Tm, and 20 s at 72 °C for 45 cycles. For each PCR, a thermal denaturing step to generate the dissociation curves was carried out to verify amplification specificity.

**ROS measurements**

Prior to ROS measurements, cells were incubated in the exactly same manner as done for transcriptome sequencing (“Experimental design for transcriptome sequencing of strain LLDDRA6 with and without Mn(II) exposure”), including four groups of Mn(II)-short, CK-short, Mn(II)-long, and CK-long. Of note, the cell density of controls (CK-short and CK-long) was commonly higher than that of Mn groups [Mn(II)-short and Mn(II)-long] owing to the ambient stress during the Mn(II) exposure” (three independent biological replicates for each group), using a TRIZol™ Max™ Bacterial RNA Isolation Kit (16096020, Invitrogen) according to the manufacturer’s instructions. Subsequently, the first-strand cDNA of RNA samples was synthesized by reverse transcription using a SuperScript IV First-Strand Synthesis System Kit (18091050, Invitrogen) according to the manufacturer’s instructions. For each PCR, 1 μL of template cDNA was mixed with 12.5 μL of 2 × SYBR Green PCR Master Mix (Roche, Basel, Switzerland) and 5 pmol of each forward and reverse primer in a final reaction volume of 25 μL. Amplification program was performed as following reaction conditions: 15 s at 95 °C, followed by 15 s at a temperature 5 °C below the primer’s true Tm, and 20 s at 72 °C for 45 cycles. For each PCR, a thermal denaturing step to generate the dissociation curves was carried out to verify amplification specificity.

**Verification of RNA-seq data by quantitative real time-PCR**

To validate the RNA-seq data, a total of 21 genes were selected from DEGs between the Mn(II)-short group and the Mn(II)-long group for qRT-PCR analyses. The primers of these 21 genes for qRT-PCR are listed in Table S2. The 16 s RNA gene (Ranieri et al. 2012) and the gyrA gene (Fan et al. 2012) were used as internal controls (primers are listed in Table S2). For the negative control, ddH₂O was used as the DNA template in qRT-PCR. The results were analyzed using the 2^−ΔΔCT method as previously described (Livak and Schmittgen 2001).

Briefly, total RNA was extracted from the cells of Mn(II)-short group and Mn(II)-long group newly incubated as instructed by the methods in “Experimental design for transcriptome sequencing of strain LLDDRA6 with and without Mn(II) exposure” (three independent biological replicates for each group), using a TRIZol™ Max™ Bacterial RNA Isolation Kit (16096020, Invitrogen) according to the manufacturer’s instructions. Subsequently, the first-strand cDNA of RNA samples was synthesized by reverse transcription using a SuperScript IV First-Strand Synthesis System Kit (18091050, Invitrogen) according to the manufacturer’s instructions. For each PCR, 1 μL of template cDNA was mixed with 12.5 μL of 2 × SYBR Green PCR Master Mix (Roche, Basel, Switzerland) and 5 pmol of each forward and reverse primer in a final reaction volume of 25 μL. Amplification program was performed as following reaction conditions: 15 s at 95 °C, followed by 15 s at a temperature 5 °C below the primer’s true Tm, and 20 s at 72 °C for 45 cycles. For each PCR, a thermal denaturing step to generate the dissociation curves was carried out to verify amplification specificity.

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used to detect ROS from cells by 2′,7′-dichlorofluorescin diacetate (DCFDA), according to the manufacturer’s instructions. The fluorescence intensity was measured at Ex/Em 488/526 nm (F-7100 Fluorescence Spectrophotometer, Hitachi, Japan).

The Superoxide Anion Assay Kit (SA-2-G, Suzhou Comin Biotechnology Co., Ltd., China) and Hydrogen Peroxide Assay Kit (H$_2$O$_2$-2-Y, Suzhou Comin Biotechnology Co., Ltd., China) were used to quantitively detect O$_2^-$ and H$_2$O$_2$ from cells, respectively, according to the manufacturer’s instructions. Before detection, cells were collected by centrifugation from cell suspensions and then resuspended in buffer solutions as supplied by the kits for subsequent ultrasonication (200 W, working time 10 s, interval time 5 s, 30 cycles) using an ultrasonic homogenizer (JY92-IIIN, Scientz, China). Therefore, O$_2^-$ and H$_2$O$_2$ detected from cells are supposed to include intracellular ones and the ones that externally attach to the membranes.

**Statistical analysis**

All statistical analyses were performed with SPSS22.0. Data were subjected to statistical evaluation using analysis of variance (ANOVA) followed by t test, with a significant level of $P < 0.05$. All data were means ± standard deviations (SD) for three independent replicates.

**Results and discussion**

**The optimum time of cell incubation and Mn(II) exposure for Mn(III) accumulation by strain LLDRA6**

The accumulation of unstable Mn(III)-intermediate is an essential, indispensable step for generation of Mn(IV) oxides from Mn(II) ions, also indicative of the occurrence of Mn(II) oxidation. To determine the effect of cell activity on Mn(III) accumulation, and when Mn(III) accumulation will occur in the presence of strain LLDRA6, trapping assays of Mn(III)–PP complexes were performed on cells with different incubation times and different Mn(II) exposure times.

Figure 1A, B, C and D presents the UV–Vis adsorption spectra of bacterial suspensions consisted of Mn(II), PP and cells at incubation times of 4 h, 8 h, 12 h, and 24 h, respectively. As a frequently used chelating agent, PP has shown to be capable of stabilizing the transient Mn(III)-intermediate, thus forming Mn(III)–PP complexes that are easily detected by UV–Vis spectrophotometer (Cui et al. 2008). It can be seen that characteristic peaks of Mn(III)–PP complexes formed under different incubation times all occur at 258 nm, which are similar to that of Mn(III)–PP complexes generated by *Pseudomonas putida* MnB1 (Thi et al. 2018). As cell incubation time increased from 4 to 24 h (Fig. 1A–D), the absorbance intensities at 258 nm gradually declined, suggesting that before Mn(II) exposure, cells with 4 h of incubation were the most active for production of Mn(III)-intermediate in comparison to cells with longer incubation time. An explanation for this trend could be that at 4 h of incubation, cells were in the mid-exponential growth phase, and then they entered in the stationary growth phase since the 12th h, as evidenced by OD$_{600}$ in the previous study (Li et al. 2020).

Furthermore, regardless of cell incubation time, 8 h of Mn(II) exposure was the optimum time for cells to accumulate Mn(III), since the corresponding absorbance intensity was always the strongest one at 258 nm. As the Mn(II) exposure time proceeded (> 8 h), the absorbances of Mn(III)–PP complexes at 258 nm unceasingly decreased, suggesting that the accumulated Mn(III) in cell suspensions was possibly oxidized to Mn(IV) oxides. It is worthy to note that at 4 h of Mn(II) exposure, the absorbance of 258 nm was far below that of 8 h of exposure, and was much closer to that of cells without Mn(II) exposure, indicating that Mn(III) accumulation by cells started at as early as the 4th h and reached the plateau at the 8th h after the cells were exposed to Mn(II).

Besides, it should be noted that cells without Mn(II) exposure had a background peak at 258 nm, not really meaning the presence of Mn(III).

Hence, the optimum time for Mn(III) accumulation by LLDRA6 at 4 h of cell incubation and 8 h of Mn(II) exposure were determined as the time parameters for cell incubation and the long-term Mn(II) exposure (i.e., $a = 4$ and $b = 8$ in “Experimental design for transcriptome sequencing of strain LLDRA6 with and without Mn(II) exposure”) in subsequent transcriptome sequencing, respectively (Fig. S2).

**Transcriptomic differences of strain LLDRA6 between the short-term and the long-term exposure of Mn(II)**

Prior to analysis of DEGs, RNA-seq data were validated by qRT-PCR. As shown in Fig. S3, for each selected gene, the log$_2$-transformed mean values obtained from qRT-PCR showed the similar trends of transcriptional upregulation or downregulation to the log$_2$-transformed fold changes from transcriptome data, suggesting the reliability of data from RNA-seq.

A total of 547 DEGs were identified between cells with short-term exposure (0.5 h) of Mn(II) [Mn(II)-short group] and cells with long-term exposure (8 h) of Mn(II) [Mn(II)-long group] (Table S4), while 1468 DEGs were identified between cells with short-term incubation (CK-short group) and cells with long-term incubation (CK-long group) in the absence of Mn(II). Venn diagram analyses further showed that out of 547 DEGs, 300 were identified as unique ones.
between Mn(II)-short and Mn(II)-long groups, indicating that they were not caused by cell growth but only due to the exposure of Mn(II) (Fig. 2A). The other 247 DEGs were shared between control groups and Mn groups, suggesting that they were not only attributed to Mn(II) exposure but also attributed to cell growth. In those 300 unique DEGs, 132 genes were upregulated and the other 168 genes were downregulated, respectively, as shown in Table S5. To better understand their potential roles in Mn(II) oxidation after cells were exposed with Mn(II), KEGG pathway enrichment analyses were performed. The top 20 of KEGG pathways with the minimum P value were shown as an enrichment bubble diagram (Fig. 2B). Notably, metabolism pathways related to aromatic or heterocyclic rings, such as phenylalanine metabolism (also known as phenylacetic acid metabolism), histidine metabolism, nitrotoluence metabolism, tyrosine metabolism, and ascorbate metabolism, were markedly enriched with Mn(II) exposure. Particularly, among them, phenylacetic acid metabolism aroused us a special attention, because its operon, consisted of 14-member phenylacetic acid genes (paa), was found to be the most transcriptionally upregulated in 547 DEGs between Mn(II)-short and Mn(II)-long groups (Tables 1 and S4).

In general, excessive Mn(II) exposure will be toxic to bacterial cells via energy metabolism deficiency or oxidative stress (Kaur et al. 2017). A recent study reported that in the highly manganese-sensitive bacterium, ΔmntP Escherichia coli, Mn(II) exposure interrupted iron–sulfur cluster and heme-enzyme biogenesis by depleting cellular iron level. Thus, disfunction of iron-dependent enzymes in the tricarboxylic acid cycle (TCA) and electron transport chain, hampered the synthesis of ATP, leading to sever energy deficiency (Kaur et al. 2017). However, in our study, KEGG analyses showed the marked enrichment of metabolism

Fig. 1 UV–Vis adsorption spectra of bacterial suspensions consisted of Mn(II), PP and cells. After 4 h (A), 8 h (B), 12 h (C), and 24 h (D) of cell incubation, respectively, 0.5 mM of Mn(II) and 0.5 mM of PP were simultaneously added in cell suspensions, with cells being constantly incubated for different times ranging from 0 to 36 h. Before detection of Mn(III)-PP complexes by UV, the cell suspension was diluted by 50-fold with ddH₂O.
pathways related to aromatic or heterocyclic rings after Mn(II) exposure (Fig. 2B), indicating that energy metabolism deficiency was not the major cause for Mn(II) toxicity to strain LLDRA6. Hence, the toxicity of Mn(II) exposure to strain LLDRA6 should be paid close attention to oxidative stress that can facilitate the generation of a high level of ROS.

In addition, according to characteristics of enzymes catalyzing for Mn(II) oxidation (including copper binding motifs and heme/Ca(II) binding domains) as reported in other bacterial species, we found only two putative MCOs-type proteins possibly responsible for Mn(II) oxidation in strain LLDRA6, i.e., CotA and laccase (Table S6), after cluster...
of orthologous groups of proteins annotation (COG) for the complete genome of LLDRA6 (Su et al. 2013; Schlosser and Höfer 2002). However, as shown in Table S6, in comparison with the short-term Mn(II) exposure, the transcriptional expressions of \textit{CotA} genes were decreased after the long-term Mn(II) exposure. For laccase, there was no marked increase for transcriptional expression in response to the long-term Mn(II) exposure. Therefore, under the current condition [50 mM of Mn(II) exposure], it cannot be determined that how much role have these MCOs-type enzymes played in the formation of Mn oxides via direct Mn(II) oxidation.

\textbf{Sharp activation of phenylacetic acid metabolism pathway hints ROS variation in LLDRA6}

Previous studies have shown that in aerobic bacteria, the complex of enzymes encoded by the \textit{paa} cluster is responsible for degradation of phenylacetic acid (PAA) and its derivatives (Teufel et al. 2010, 2012; Cook 2019). Intriguingly, after exhaustingly searching for relevant literatures, we find that phenylacetate catabolism is also important for resisting killing by \textit{H}_2\textit{O}_2 in bacteria (Green et al. 2020). A \Delta \textit{paa} mutant of \textit{Acinetobacter baumannii}, which lacks part of the phenylacetate degradation pathway, was found to be more susceptible to \textit{H}_2\textit{O}_2 killing than a wild-type strain (Green et al. 2020). The authors suggest that phenylacetate degradation may only be required to withstand environmental \textit{H}_2\textit{O}_2 encountered at high concentrations (\(\geq 40\) mM) (Green et al. 2020). Herein, we supposed that for strain LLDRA6, the drastically upregulated expression of the \textit{paa} cluster may attach to the variation of ROS contents in cells, due to the stress of ambient Mn(II) ions. Therefore, we further qualitatively analyzed variation of ROS contents for strain LLDRA6 with and without the exposure of Mn(II).

\textbf{ROS variation in LLDRA6 with and without Mn(II) exposure}

2',7'-Dichlorofluorescin diacetate (DCFDA), capable of freely penetrating the cell membrane into the cytoplasm, is commonly used as a fluorometric probe for qualitative detection of various ROS in cells (Fan and Li 2014). Since LB media were able to produce a certain amount of ROS (Vasiljevic et al. 2020), they were removed by centrifugation before detection of ROS by DCFDA. As shown in Fig. 3, fluorescent characteristic peaks of ROS produced by cells occurred at 526 nm in the range of 510–650 nm. No matter which the Mn(II) exposure time was (0.5 or 8 h), the fluorescence intensities of cells with exposure of 50 mM Mn(II) were stronger than that of cells with no exposure of Mn(II), at an equivalent level of cell density (\text{OD}_{600} of 0.9 for the short-term exposure group, and \text{OD}_{600} of 1.7 for the long-term exposure group), suggesting a significant variation of ROS contents in strain LLDRA6 with and without the exposure of Mn(II).

\textbf{Evidence for Mn(II) oxidation by LLDRA6 via superoxide generation coupled to hydrogen peroxide consumption}

Commonly, ROS are short-lived oxygen radicals generated in the reduction of oxygen to water via the electron
transfer chains, including O$_2^-$, H$_2$O$_2$, O$_2$, HO$_2^-$, HO$_-$, ROO$_-$, and RO$_-$, etc. (Hansel et al. 2019; Fan and Li 2014). As described by the reaction Eq. (1), since O$_2^-$ and H$_2$O$_2$ play a key role in indirect oxidation of Mn(II), their contents in cells were specifically tested with and without Mn(II) exposure.

As shown in Fig. 4A, LB medium was able to generate a large amount of O$_2^-$ by itself, even producing two-fold greater amount of O$_2^-$ in the presence of 50 mM of Mn(II). Therefore, LB medium was removed by centrifugation prior to detection of O$_2^-$ produced by cells.

Cells from both CK-short and CK-long groups were able to produce O$_2^-$ (Fig. 4A). Interestingly, although the cell density of CK-long (OD$_{600} = 1.7$) was nearly two times greater than that of CK-short (OD$_{600} = 0.9$), the O$_2^-$ concentration of cells from CK-long (2.19 μM) was much lower than that of cells from CK-short (5.26 μM). The similar trend of an inverse relationship between cell density and O$_2^-$ production, has also been observed in other bacteria (Díaz et al. 2013; Hansel et al. 2019). These studies show that O$_2^-$ concentrations steadily increased during active growth but then

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**Fig. 3** ROS fluorescence intensities of cells with exposure of 50 mM Mn(II) for 0.5 h (Mn(II)-short), cells with exposure of 50 mM Mn(II) for 8 h (Mn(II)-long), cells without Mn(II) exposure for 0.5 h (CK-short), and cells without Mn(II) exposure for 8 h (CK-long), respectively, in the range of 510–650 nm.

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**Fig. 4** Evidence for that superoxide generated only by living cells is the oxidant for Mn(II) oxidation. A Superoxide concentrations of LB liquid medium, living cells, dead cells, and living cells incubated with SOD, respectively. Asterisk (*) denotes the significant difference at $P < 0.05$ level using independent samples t test. B Experimental treatments marked with specific symbols as shown in (A) were repeated, only except for addition of 0.5 mM of Mn(II) instead of 50 mM of Mn(II) for Mn(II) exposure. The equal amounts of Mn(II) and PP (0.5 mM) were simultaneously added into the cell suspension for 8 h of incubation. After that, the cell suspension was diluted by 50-fold with ddH$_2$O for detection of Mn(III)–PP complexes by UV. C Cell suspensions marked with specific symbols from (A) were continued to incubate for 4 days, and then mixed with 0.04% LBB ($V_{cell}: V_{LBB} = 1:5$) for staining.
declined upon entering stationary phase, indicating their potential involvement in cell signaling except for simple responses to oxidative stress within microbial or environmental systems (Hansel et al. 2019).

When the cells were exposed to 50 mM of Mn(II) for 0.5 h, O$_2^\cdot$ concentration of cells from Mn(II)-short (5.06 μM) slightly declined compared to that of cells from CK-short (5.26 μM), indicating a rapid involvement of O$_2^\cdot$ in Mn(II) oxidation. As the Mn(II) exposure time increased to 8 h, O$_2^\cdot$ concentration of cells from Mn(II)-long significantly increased in comparison to that of cells from CK-long (from 2.19 to 2.88 μM). This is because strain LLDRA6 was in middle of oxidizing Mn(II) ions when a vast amount of O$_2^\cdot$ were needed to constantly produce, as evidenced by the fact that 8 h of Mn(II) exposure was the optimum time for cells to accumulate Mn(III) (Fig. 1).

However, it cannot be ignored that substances other than living cells (e.g., LB media, Mn(II) ions and dead cells), were also capable of producing O$_2^\cdot$ during incubation (Fig. 4A). To confirm that O$_2^\cdot$ only generated by living cells is the true oxidant for Mn(II) oxidation, Mn(III)-trapping and leucoberbelin blue (LBB) stain assays (Krumbein and Altmann 1973) were tested for living cells, LB media, and dead cells.

As shown in Fig. 4B, no absorption peak of Mn(III)—PP complexes could be found at 258 nm for LB medium in the absence and presence of Mn(II), showing that O$_2^\cdot$ produced by the medium was ineffective in oxidizing Mn(II). In addition, dead cells in LB medium with or without addition of Mn(II) were not capable of producing Mn(III)—PP complexes after 8 h of incubation, since no characteristic peaks were detected at 258 nm. By comparison, living cells grown in LB medium with addition of Mn(II) produced a much stronger characteristic peak at 258 nm than living cells grown in the absence of Mn(II) did (considered as the background peak). Then, the strong characteristic peak produced by living cells grown in the presence of Mn(II), markedly attenuated as excessive superoxide dismutase (SOD) was used to scavenge superoxide. These results suggest that O$_2^\cdot$ only produced by living cells were involved in the formation of Mn(III)—PP complexes after 8 h of cell incubation. More importantly, after 4 days of continuous incubation, only the suspension of living cells incubated in LB medium with addition of Mn(II) showed deep blue in LBB stain assays (Fig. 4C), confirming the presence of Mn oxides. Thus, these results demonstrate that O$_2^\cdot$ only generated by living cells is the true oxidant for Mn(II) oxidation.

It is generally accepted that the production of O$_2^\cdot$ alone does not ensure the formation of Mn oxides in indirect Mn(II) oxidation, since H$_2$O$_2$ produced by cells may facilitate the reduction of Mn(III) to Mn(II) (Andeer et al. 2015; Lingappa et al. 2019). For instance, a marine alpha-proteobacterium, Ruegeria sp. TM1040, does not generate Mn oxides despite its rapid production rates of extracellular O$_2^\cdot$ (Diaz et al. 2013; Learman and Hansel 2014).

As shown in Fig. 5, the concentration of H$_2$O$_2$ generated by cells after 0.5 h exposure of Mn(II) (Mn(II)-short) was much higher than that produced by cells from the control group (CK-short), owing to the rapid response to oxidative stress. As the exposure time increased to 8 h, the H$_2$O$_2$ concentration of Mn(II)-long cells significantly lowered in comparison to that of cells with no exposure of Mn(II) (CK-long), confirming a heavy consumption of H$_2$O$_2$ (from 25.99 to 22.87 μM), which facilitated to pull the equilibrium of the reaction Eq. (1) towards accumulation of Mn(III). A possible explanation for the consumption of H$_2$O$_2$ in cells of Mn(II)-long group, could be that the gene encoding catalase was significantly upregulated in transcription by 1.08-fold after the long-term Mn(II) exposure, as shown in Table S7, thus resulting in acceleration for decomposition reaction of H$_2$O$_2$.

Overall, these results suggest that Providencia sp. LLDRA6 exploited the indirect Mn(II) oxidation strategy of O$_2^\cdot$ production coupled to H$_2$O$_2$ consumption to form Mn oxides.

**Prediction of links between ROS and phenylacetic acid metabolism pathway**

Figure 6 presents a schematic diagram for the proposed process of Mn oxides generation by Providencia sp. LLDRA6. Regardless of the absence and presence of direct enzymatic oxidation in LLDRA6 (Table S6), the formation of extracellular Mn oxides by LLDRA6, as evidenced by our previous work (Li et al. 2020), now can be partially attributed to the attack of O$_2^\cdot$ that externally attached to cell membranes to Mn(II) (Learman et al. 2011b; Learman and Hansel 2014; Andeer et al. 2015).

Once strain LLDRA6 is exposed to 50 mM of Mn(II), excessive Mn(II) will be harmful to the cell viability, as previously evidenced by OD$_{600}$ (Li et al. 2020), through facilitating a higher level generation of ROS due to heavy-metal induced oxidative stress (Fig. 3) (Todsapol et al. 2020; Niu et al. 2020). ROS are often produced as by-products during the reduction of oxygen to water in electron transport chains (Hansel et al. 2019). Thus, enzyme complexes for respiration anchored in cell membrane can produce a large amount of extracellular O$_2^\cdot$ to react with Mn(II) to generate extracellular Mn oxides. In addition, some proteins, such as AHPs, malate dehydrogenase and xanthine dehydrogenase, are reported to take part in production of extracellular O$_2^\cdot$ via transmembrane transport (Andeer et al. 2015).

After the long-term exposure of Mn(II), the transcriptional level of the paa cluster is drastically upregulated (Table 1), indicating that PAA or PAA-like derivatives sharply accumulates and needs to be degraded as soon as possible, because from a healthy perspective, the excessive
aromatic compounds are harmful to cell vitality (Teufel et al. 2010). Therefore, the question arises, why does the paa cluster be transcriptionally activated in the long-term presence of Mn(II). In other words, what the link would be between the increment of ROS and the sharp activation of PAA catabolic pathway. According to the detailed information from catabolic pathway of PAA in aerobic bacteria as reported by Teufel et al. (2010), we find that the crucial process for degrading the inert aromatic ring of PAA, is the introduction of an oxygen atom into the aromatic ring catalyzed by the multicomponent enzyme PaaABCDE, and then the subsequent formation of a highly reactive oxygen-containing, seven-member heterocycle (namely, oxepin) catalyzed by PaaG (Fig. 6). Furthermore, the ring of the C–O seven-member heterocycle is cleaved and opened at the oxygen site catalyzed by PaaZ.

Therefore, for inert, recalcitrant aromatic rings, the formation of highly reactive oxepins via epoxidation is an elaborate strategy for ring cleavage (Teufel et al. 2010). According to this conclusion, possible links between the increment of ROS and the sharp activation of phenylacetic acid catabolism can be explained by three reasons as follows. (1) Commonly, the oxygen (O₂) is used for epoxidation of the aromatic ring of PAA catalyzed by PaaABCDE. During the long-term exposure of Mn(II), the excessive ROS replaced the oxygen to involve in epoxidation, speeding up the catabolic pathway of PAA. (2) In fact, PAA catabolic pathway is not restricted to phenylacetate (Teufel et al. 2010). Other PAA-like aromatic compounds (e.g., benzoate, phenylalanine and styrene) can also enter in the same pathway for their degradation (Teufel et al. 2010). Hence, these PAA-like aromatic compounds were also attacked by ROS to form highly reactive oxepins, thus increasing their concentrations. Though exhibiting high chemical activity, oxepins are poisonous to cells, which need to be immediately hydrolyzed (Teufel et al. 2010), thereby further activating the PAA catabolic pathway. (3) The excessive oxygen radicals resulted in a variety of oxygen-containing heterocycles that are similar to oxepins (oxepin-like ones) by attacking non-aromatic compounds (e.g., aliphatic hydrocarbons) (Meng et al. 2018), which in turn upregulated the transcriptional expression of the paa cluster.

Taken together, PAA, PAA-like derivatives and oxepin-like oxygen-containing heterocycles, were all crowded in this catabolic pathway (Teufel et al. 2012), as a result of the response to Mn(II)-induced oxidative stress, consequently accelerating the transcription of the paa cluster. As to why does the bacterium choose PAA catabolic pathway for detoxification, a possible explanation is that all intermediates in this pathway are combined with CoA, which can be recognized rapidly and bound through CoA-binding motives of the processing enzymes (Teufel et al. 2010). More importantly, the final products of PAA catabolic pathway, acetyl-CoA and succinyl-CoA, are able to enter in the tricarboxylic acid cycle for safe energy conservation.
Conclusions

In this work, the mechanism of Mn(II) oxidation by a soil-borne bacterium Providencia sp. LLDRA6 under high Mn(II) concentration (50 mM) was explored. Results from transcriptome sequencing for LLDRA6 brought an important clue to unravel the ROS-dependent behavior of Mn oxides production by LLDRA6, i.e., the sharply transcriptional upregulation of genes for phenylacetic acid catabolism, which may suggest a significant variation of ROS in cells during Mn(II) exposure. Chemical activity assays for ROS confirmed that LLDRA6 exploited the indirect Mn(II) oxidation strategy of O$_2^-$ production coupled to H$_2$O$_2$ consumption to form Mn oxides. During Mn(II) exposure, the excessive ROS, generated in response to oxidative stress caused by Mn(II), transcriptionally activated genes for phenylacetic acid catabolism presumably by increasing concentrations of highly reactive oxepins.

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Fig. 6 Proposed schematic diagram for processes of Mn oxides formation by Providencia sp. LLDRA6. During the exposure of 50 mM of Mn(II), a large amount of various ROS were produced in cells due to heavy-metal induced oxidative stress. Among them, superoxide oxidized Mn(II) to form extracellular Mn oxides. Meanwhile, the transcriptional level of the paa cluster was found to be drastically upregulated during Mn(II) exposure. This is presumably because excessive ROS replaced oxygen (O$_2$) to participate in the formation of the C-O seven-member heterocycles (namely, oxepins, as noted in red) which were the key intermediates in the phenylacetic acid catabolic pathway. Phenylacetic acid catabolic pathway was quoted from Teufel et al. (2010) (color figure online)

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Author contributions DL conceived and designed the study. SC and DL retrieved the literature and drafted the manuscript. ZD, JC, JL, XR, ZL, FL and JH participated in the study design and editing of the manuscript. All authors read and approved the final manuscript.

Data availability The genome sequence included in the study has been submitted to GenBank with the accession number CP067099. The clean reads of genome sequencing are deposited in the NCBI Sequence Read Archive under BioProject PRJNA724814. The RNA-seq data are deposited in the NCBI Sequence Read Archive under BioProject PRJNA690198.

Declarations

Conflict of interest The authors declare no conflicts of interest.
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