ORIGINAL ARTICLE

Microbe–microbe interactions trigger Mn(II)-oxidizing gene expression

Jinsong Liang[1,2,4], Yaohui Bai[1,4], Yujie Men[3] and Jiuhui Qu[1]

[1]*Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China;* [2]*University of Chinese Academy of Sciences, Beijing, China and* [3]*Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

Manganese (Mn) is an important metal in geochemical cycles. Some microorganisms can oxidize Mn (II) to Mn oxides, which can, in turn, affect the global cycles of other elements by strong sorption and oxidation effects. Microbe–microbe interactions have important roles in a number of biological processes. However, how microbial interactions affect Mn(II) oxidation still remains unknown. Here, we investigated the interactions between two bacteria (*Arthrobacter* sp. and *Sphingopyxis* sp.) in a co-culture, which exhibited Mn(II)-oxidizing activity, although neither were able to oxidize Mn(II) in isolation. We demonstrated that the Mn(II)-oxidizing activity in co-culture was most likely induced via contact-dependent interactions. The expressed Mn(II)-oxidizing protein in the co-culture was purified and identified as a bilirubin oxidase belonging to strain *Arthrobacter*. Full sequencing of the bilirubin oxidase-encoding gene (*boxA*) was performed. The Mn(II)-oxidizing protein and the transcripts of *boxA* were detected in the co-culture, but not in either of the isolated cultures. This indicate that *boxA* was silent in *Arthrobacter* monoculture, and was activated in response to presence of *Sphingopyxis* in the co-culture. Further, transcriptomic analysis by RNA-Seq, extracellular superoxide detection and cell density quantification by flow cytometry indicate induction of *boxA* gene expression in *Arthrobacter* was co-incident with a stress response triggered by co-cultivation with *Sphingopyxis*. Our findings suggest the potential roles of microbial physiological responses to stress induced by other microbes in Mn(II) oxidation and extracellular superoxide production.
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Introduction

Interspecific interactions occur extensively among coexisting microbes, which often generate enhanced or emergent biochemical properties (Li and Gu, 2007; Wintermute and Silver, 2010). Hence, these interactions might promote the biotransformation of substrates (Li and Gu, 2007; Kimura and Okabe, 2013; Li *et al.*, 2015) and increase the biogeochemical cycle rate of involved elements. Various microbial interspecific interactions have been discovered in laboratory cultures and natural environments, for example, metabolite exchange (Li and Gu, 2007; Wintermute and Silver, 2010; Kimura and Okabe, 2013; Zelezniak *et al.*, 2015), interspecies hydrogen/electron transfer (Walker *et al.*, 2009; Summers *et al.*, 2010; Men *et al.*, 2012), quorum sensing (Miller and

Bassler, 2001; Gupta and Schuster, 2013), as well as activation of silent genes (Schroeckh *et al.*, 2009; Netzker *et al.*, 2015). Growing evidence suggests their important roles in shaping microbial communities (Miller and Bassler, 2001; Zelezniak *et al.*, 2015), inducing the production of secondary metabolites (Nutzmann *et al.*, 2011; Onaka *et al.*, 2011) and biotransforming organic substances (Li and Gu, 2007; Kimura and Okabe, 2013; Li *et al.*, 2015). However, their contributions to metal transformations remain largely unknown.

Microbial Mn(II) oxidation is an important biogeochemical process in the global cycles of many other elements (for example, trace metals, C, N, S) because of the oxidation (Sunda and Kieber, 1994; Forrez *et al.*, 2010) and adsorption (Villalobos *et al.*, 2005; Toner *et al.*, 2006) properties of generated manganese (Mn) oxides (III and IV). The production of widespread Mn oxides in the natural environment can be largely attributed to microbes, as they can enzymatically accelerate homogeneous Mn(II) oxidation by O_2 by several orders of magnitude (Morgan, 2005). Based on isolated Mn(II)-oxidizing species, it has been demonstrated that multicopper oxidases (MCOs) (Dick *et al.*, 2008a; Su *et al.*, 2013, 2014) and

peroxidases (Anderson *et al.*, 2009; Andeer *et al.*, 2015) are involved in Mn(II) oxidation. Previous studies have focused more on identification of Mn(II)-oxidizing bacteria by isolation of pure cultures. However, the influence of biological interactions on Mn(II) oxidation has not been well understood.

In this study, we explored the mechanism of a novel 'cooperative Mn(II) oxidation' phenomenon between two bacterial strains, *Arthrobacter* sp. QXT-31 (Gram-positive, aerobic, subsequently referred as *Arthrobacter*) and *Sphingopyxis* sp. QXT-31 (Gram-negative, aerobic, subsequently referred as *Sphingopyxis*), and neither is capable of oxidizing Mn(II) in isolation (Liang *et al.*, 2016). We purified and identified a novel Mn(II)-oxidizing protein produced in co-culture, fully sequenced the encoding gene, and explored possible biological interactions that caused induction of the Mn(II)-oxidizing activity in co-culture of the two strains.

Materials and methods

Strain isolation and 16S ribosomal RNA gene analysis
The strains used in this study, *Arthrobacter* sp. QXT-31 (subsequently referred as strain *Arthrobacter*) and *Sphingopyxis* sp. QXT-31 (subsequently referred as strain *Sphingopyxis*) (Liang *et al.*, 2016), were isolated from surface soil of the Xiangtan manganese mine (Hunan Province, China, N27°58', E112°51') using a modified peptone-yeast extract-glucose (mPYG) medium (Adams and Ghiorse, 1985; Liang *et al.*, 2016) buffered by 10 mм 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2), with the sterile addition of 100 µM $MnCl_2$. After quadruplicate subculturing, the suspension was spread onto solid mPYG medium containing 100 µM $MnCl_2$, and incubated at 30 °C for 5 days. Brownish Mn oxides appeared where two different-colored (yellow and white) colonies overlapped. The two colonies were then subjected to further purification to obtain pure cultures. The Mn oxides produced by the two strains co-culture were confirmed and quantified using the leucoberbelin blue (LBB) method (Krumbein and Altmann, 1973). The 16S rDNA sequence fragments (>1300 bp) of the two strains were amplified, sequenced and identified by BLAST searching (Method S1).

Co-cultivation and cell density quantification
The two strains were first pre-cultured in isolation for 24 h, then mixed with an equivalent volume, forming a co-culture. The co-culture was then used as inoculum (3% (v/v) subculturing) for cell density quantification, or directly cultivated for protein and RNA extraction, and extracellular superoxide detection.

A viable count was used to measure cell numbers during co-cultivation (3% (v/v) subculturing). Cultures were homogenized by vigorous shaking

then serially diluted and immediately spread onto solid mPYG medium. Individual white (*Arthrobacter*) and yellow (*Sphingopyxis*) colonies were formed, indicating that there were no aggregated cells of the two strains in the inoculum. The colony-forming units (CFUs) of each strain were determined to represent cell densities.

To distinguish counts of living and dead cells, cells in co-cultures (3% (v/v) subculturing) were also assayed with a BD Influx cytometry (BD Biosciences, San Jose, CA, USA). Sterile ascorbic acid (250 µM, 2.5 times the concentration of initial Mn(II)) was added to all samples to completely reduce Mn oxides (if any), and thorough mixing with a pipette was done to eliminate cell aggregates. The cells were then stained with two florescent nucleic acid stains (SYTO9 and propidium iodide) from a LIVE/DEAD BacLight Bacterial Viability and Counting Kit (L34856, Thermo Fisher Scientific, Eugene, OR, USA). Cells of *Arthrobacter* and *Sphingopyxis* were distinguished by their different light scatter properties. The cell densities of each strain (living and dead, based on the criteria in the kit) were quantified by adding the internal microsphere counting standard.

Protein(s) fractionation
Total proteins were prepared in three fractionations:

(1) Secreted proteins. Monocultures of each strain were first cultured for 24 h without Mn(II), and then mixed for co-cultivation. After 3 h of co-cultivation, the cells in 1 liter of medium were harvested by centrifugation (10 000 *g*, 10 min, 4 °C), and the spent culture medium was filtered through a 0.22-µm filter (JINTENG, Tianjin, China) and then ultrafiltered to 10 ml using a Millipore stirred filtration cell (Millipore, Darmstadt, Germany) with a 10-kDa nominal molecular mass limit (NMWL) filter.

(2) Loosely bound outer membrane (LBOM) proteins. The cell pellets from the last centrifugation of (1) were resuspended in 50 ml of chaotropic salt buffer (400 mм $MgCl_2$ and 30 mм Tris/HCl, pH 7.5) and gently stirred for 8–12 h on ice. After centrifugation (10 000 *g*, 10 min, 4 °C), the supernatant was collected and filtered with a 0.22-µm filter, then ultrafiltered with a 10-kDa NMWL Amicon Ultra centrifugal filter (Milipore, Cork, Ireland) to 1 ml.

(3) Intracellular proteins (represented by a partial crude extract). Cell pellets from (2) were resuspended in lysis buffer (30 mм Tris/HCl, pH 7.5; 50 mм NaCl; 1 mм phenylmethanesulfonyl fluoride; 5% glycerol) and lysed by a JN-02C French press (JNBIO, Guangzhou, China) at 1950 bar (seven rounds) at 4–6 °C. The lysis solution was centrifuged for 30 min at 12 000 *g* (4 °C) and filtered with a 0.22-µm filter.

All secreted proteins, LBOM proteins and 1 ml of the partial crude extracts were first dialyzed against 50 mM Tris-HCl (pH 6.8) for native polyacrylamide gel electrophoresis (native-PAGE) or slightly modified blue native-PAGE (BN-PAGE) solubilization buffer (50 mM NaCl, 50 mM imidazole/HCl, and 2 mM 6-aminohexanoic acid, pH 7.0) (Wittig *et al.*, 2006) for BN-PAGE for 12 h at 4 °C, with buffer replaced every 4 h, then concentrated to 1 ml with a 10-kDa NMWL Amicon Ultra centrifugal filter.

Blue native-PAGE
Extracted proteins were subjected to the following pre-treatment for BN-PAGE, as per Wittig *et al.* (2006) with several modifications. First, 20% detergent dodecylmaltoside (DDM) solution was added at a ratio of 3:100 (DDM solution:protein solution, v-v) and allowed to solubilize for 10 min at room temperature. Next, the mixture was centrifuged for 15 min at 12 000 *g* (4 °C), and 50% glycerol was added to the supernatant at a ratio of 20:100 (50% glycerol:protein solution, v-v). Finally, 5% Coomassie blue G-250 dye stock suspension (dissolved in 500 mM 6-aminohexanoic acid) was added at a ratio of 1.5:100 (Coomassie blue G-250 dye stock:protein solution, v-v). The mixture was allowed to equilibrate at room temperature for 10 min before sample loading (40 µl into each sample well). A 6.5–10% acrylamide gradient gel was cast for BN-PAGE. BN-PAGE was performed in a cold room (4 °C). During the first 30 min, cathode buffer B (50 mM tricine, 7.5 mM imidazole, and 0.02% Coomassie blue G-250, pH 7.0) was used, and the voltage was set at 100 V. The current was then limited to 18 mA per gel (0.1 × 13 × 10 cm), and the voltage was limited to 500 V. After 60 min, cathode buffer B was replaced by a slightly blue cathode buffer B/10 (50 mM tricine, 7.5 mM imidazole, and 0.002% Coomassie blue G-250, pH 7.0), and electrophoresis was continued for 3.5 h.

In-gel Mn(II) oxidation assay and Coomassie blue staining
After electrophoresis, the gel was first removed from the glass plates and transferred into a container filled with 300 ml of Milli-Q water to destain the Coomassie dye in gel. The container was put on a horizontal shaker for efficient destaining, and the spent water was replaced with fresh Milli-Q water every 30 min until the blue color was not visible. The gel was then transferred to 300 ml of buffer (100 µM MnCl₂, 10 mM HEPES, pH 7.6) and incubated at 30 °C in the dark until brownish Mn oxides appeared. The gel was then visualized on a gel scanner.

After in-gel Mn(II) oxidation assay, the gel was immersed into 0.1% (w/v) ascorbic acid solution to dissolve the brownish Mn oxides to avoid their possible interference in the following staining. The gel was washed three times with 300 ml of Milli-Q water for 20 min on a horizontal shaker, and then transferred to 200 ml of Coomassie G-250 staining solution. The gel was stained for 12 h, then rinsed with Milli-Q water, and finally destained for 1 h with 200 ml of destaining solution. The destaining procedure was repeated with fresh destaining solution until the gel background became colorless. The gel was then visualized again on a scanner.

Protein identification
The Mn(II)-oxidizing protein band was excised from the gel, digested with trypsin and then analyzed by nano liquid chromatography-MS/MS (nanoLC-MS/MS). The protein data related to the genera *Arthrobacter* and *Sphingopyxis* in the non-redundant protein database of the NCBI were downloaded (on 18 May 2016) as the reference sequence database. The MS/MS spectra of the Mn(II)-oxidizing protein were searched against the reference database using an in-house Proteome Discoverer searching algorithm. The full method is described in Method S3.

Full-length sequencing of the Mn(II)-oxidizing gene
To obtain the full sequence of the Mn(II)-oxidizing gene, PCR primers were designed (Supplementary Table S1), taking the gene sequence encoding the best matched protein in MS/MS identification as the target sequence, to amplify the partial sequences of the Mn(II)-oxidizing gene. Genomic walking was then adopted to amplify the remaining sequence (primers in Supplementary Table S1). The amplified fragments of the Mn(II)-oxidizing gene were sequenced, trimmed, and assembled with DNAMAN (version 6.0) to obtain the full gene sequence.

RNA extraction, sequencing and transcriptomic analysis
Monocultures of the two strains were first cultivated for 24 h with 100 µM of MnCl₂, and then mixed for co-cultivation. One cell sample was taken at 0 h of co-cultivation, and two cell samples from each of the two biological replicates were taken at 1.5, 3, 4 and 7 h of co-cultivation. Cell pellets were collected by centrifugation (10 000 *g*, 3 min, 4 °C), and were subjected to RNA extraction immediately using TRNzol Reagent (TIANGEN, Beijing, China) according to the manufacturer's instructions, with modification in the cell lysis step, where cell pellets were pulverized by a pestle in liquid nitrogen. RNA integrity was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and only samples with RNA integrity numbers above 7.5 were selected for later treatment. RNA was stored at −80 °C before complementary DNA library construction.

Two types of complementary DNA libraries were prepared and sequenced using an Illumina HiSeq

2000 sequencing system (Agilent Technologies, San Diego, CA, USA). Single-end libraries (49 bp in length) were prepared for all nine RNA samples. To reconstruct the transcript sequences, a paired-end library (90 bp in length) was prepared for a mixture of all RNA samples after duplex-specific nuclease normalization (Christodoulou *et al.*, 2011).

The sequence reads generated from the paired-end library were assembled by the short-reads assembly program Trinity (Haas *et al.*, 2013) for transcripts generation. The 49-base reads from the nine single-end libraries were aligned to the transcripts and the abundance of all transcripts at specific culturing time was estimated using the built-in Perl script (*align_and_estimate_abundance.pl*) with the RNA-Seq by expectation maximization method (Li and Dewey, 2011). A matrix of normalized expression values was then constructed using the built-in Perl script (*abundance_estimates_to_matrix.pl*) with the trimmed mean of M-values normalization method. The Mn(II)-oxidizing gene transcript abundance in each single-end library was calculated by normalizing the number of reads aligned to the Mn(II)-oxidizing gene sequence to the total number of reads aligned to all assembled transcript sequences.

Differential expression analysis was performed by edgeR (Robinson *et al.*, 2010) using the built-in command (*run_DE_analysis.pl*) in the Trinity pipeline. The differentially expressed genes were then extracted and clustered using the built-in Perl script (*analyze_diff_expr.pl*), with a false discovery rate < 0.001 and minimal $\log_2$ ratio of 1.585 (default value is 2). The differentially expressed genes were clustered according to their expression patterns using the built-in Perl script (*define_clusters_by_cutting_tree.pl*) with the Ptree (value 20%) method.

Functional annotation of the transcripts was completed with Blast2GO (Pro version, 3.1) software (Conesa *et al.*, 2005).

Exploring contributions of cells, cell-free filtrate (CFF), and cell lysates to Mn(II)-oxidizing activity
Monocultures of each strain were cultivated for 24 h with 100 μM of $MnCl_2$, and then subjected to centrifugation at 3500 *g* for 10 min at 4 °C. The supernatant was filtered twice with a 0.22-μm filter and was regarded as a CFF. The cells were washed twice with solution A (0.5 g l^{-1} $MgCl_2 \cdot 7H_2O$; 60 mg l^{-1} $CaCl_2 \cdot 2H_2O$; 10 mM HEPES, pH 7.2), and deposited again by centrifugation at 3500 *g* for 10 min at 4 °C. The cells or CFF of one monoculture were added to culture suspension of the other monoculture for a total of four combination scenarios. Besides, CFF of the co-culture after the initiation of Mn(II) oxidation was added to the *Arthrobacter* and *Sphingopyxis* monocultures, respectively. The produced Mn oxides after 30-h cultivation were quantified using the leucoberbelin blue method.

For lysate preparation, *Arthrobacter* and *Sphingopyxis* monocultures were first separately cultivated for 24 h with 100 μM of $MnCl_2$. The cells were then collected by centrifugation (3500 *g*, 10 min, 4 °C) and resuspended in a smaller volume of culture medium of each monoculture, resulting in 30 times concentrated culture suspension. The concentrated cell suspensions of *Arthrobacter* (Gram-positive, thick cell wall) and *Sphingopyxis* (Gram-negative, thin cell wall) were lysed at 4–6 °C through a French press at 1950 bar (seven rounds) and 1350 bar (three rounds), respectively. The lysates of each strain were first centrifuged at 12 000 *g* for 30 min at 4 °C, and then filtered through two tandem 0.22-μm filters. The filtered lysate of one monoculture was added to a 24-h pre-grown culture (with 100 μM of $MnCl_2$) of the other monoculture. The produced Mn oxides were determined after 30 h.

Extracellular superoxide detection
Monocultures of each strain were first cultured for 24 h without Mn(II), and then mixed for co-cultivation. Extracellular production of superoxide in the co-cultures, as well as in the pre-grown monocultures of each strain, was detected using a superoxide-specific chemiluminescence (CL) probe MCLA (2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3(7H)-one; TCI, Tokyo, Japan) (Godrant *et al.*, 2009) by an Infinite M200 Pro microplate reader (Tecan, Salzburg, Austria). At predetermined culture times, bacteria suspensions in co-cultures and monocultures were added respectively to three wells (280 μl per well) in a white 96-well plate, and the CL signal from bacteria suspensions without probe MCLA were first read. Then, 6 μl MCLA, maintained at room temperature in the dark, was added to wells containing bacteria suspensions and thoroughly mixed with a pipette (for a final MCLA concentration of 2.5 μM). CL emission from each well was read for 15.5 min using an acquisition time of 1 s per well. Finally, 50 kU l^{-1} superoxide dismutase from bovine erythrocytes (Sigma-Aldrich, St Louis, MO, USA) was added to scavenge superoxide to prevent its reaction with MCLA probe, and CL emission from each well was read for an additional 3 min. The average values of last 10 CL signal values (in stable stage; after MCLA addition) before and after superoxide dismutase addition in each well were calculated, and taken as the CL intensity of each sample after the average CL signal values before MCLA addition were subtracted.

Accession numbers
The 16S rDNA sequences of *Arthrobacter* and *Sphingopyxis* and the full sequence of gene *boxA* were deposited in GenBank under accession numbers KC765085, KC765086 and KT692935, respectively. All raw sequences generated from RNA-Seq

were deposited in the NCBI Sequence Read Archive database under accession number PRJNA290005.

Results and Discussion

Mn(II)-oxidizing activity was induced as a temporary response in Arthrobacter and Sphingopyxis co-culture
When Mn(II)-oxidizing microorganisms were isolated from the surface soil of the Xiangtan manganese mine using solid mPYG medium containing $MnCl_2$, we observed that brownish Mn oxides only appeared where two different colonies (one white, one yellow) overlapped (Figure 1a). The two bacterial strains were further isolated from the white and yellow colonies, and were identified and designated as *Arthrobacter* sp. QXT-31 and *Sphingopyxis* sp. QXT-31, respectively.

No Mn(II) oxidation occurred in the monoculture of either strain in liquid medium, even after 1 month of incubation when both strains were no longer growing, as the optical densities at 600 nm decreased in the monocultures (Supplementary Figure S1). In contrast, in the co-culture, Mn(II) oxidation occurred at 20 h and 85% of added Mn(II) was oxidized at 30 h (Figure 1b). The incomplete Mn(II) oxidation by the co-culture might be from the inaccessibility of Mn(II) adsorbed onto formed Mn oxides to Mn(II)-oxidizing factors. Similar to most single strains of Mn(II)-oxidizing bacteria, Mn(II) oxidation in the co-culture did not occur during cell growth phase, but at the time point when living cell densities of each strain reached maximum density (Figure 1b). Mn(III) was detected as an intermediate during Mn(II) oxidation by co-culture in the liquid medium (Supplementary Method S2 and Supplementary Figure S2), which is consistent with the pathway of Mn(II) oxidation by single species (Tebo *et al.*, 2005; Anderson *et al.*, 2009).

To determine whether or not Mn(II)-oxidizing single strain was generated after co-cultivation, the Mn(II)-oxidizing co-culture (24 h) was diluted and then spread onto solid mPYG medium containing 100 μM of $MnCl_2$. No brownish Mn oxides were observed around single colonies (photo not shown), indicating that neither strain was capable of independent Mn(II) oxidation, even when re-isolated from an active co-culture. Taken together, we demonstrated that Mn(II)-oxidizing activity could only be induced in the presence of both strains, and was a temporary biological response to interactions between the two strains.

Similar findings between species belonging to *Arthrobacter* (formerly *Corynebacterium*) and *Flavobacterium* (formerly *Chromobacterium*) have been reported (Bromfield and Skerman, 1950; Bromfield, 1956; Clement, 2006). This suggests that Mn(II) oxidation caused by interspecific interactions in microbial consortia is not restricted to certain species. Instead, it might be more commonly occurring in the environmental communities.

Mn(II)-oxidizing gene was silent in Arthrobacter and activated in response to presence of Sphingopyxis
Experiments were conducted to look at two possibilities of Mn(II) oxidation between the two strains: (i) a co-operative mechanism where factors from both strains are required for the catalytic mechanism, or (ii) one strain is sufficient for the catalytic mechanism, but factors from the other strain are required to induce it.

(1) Location of Mn oxides on cell surface. We first addressed which strain carried out Mn(II) oxidation reaction by looking at the location of Mn oxides on cell surface. The morphology of the cell surface of
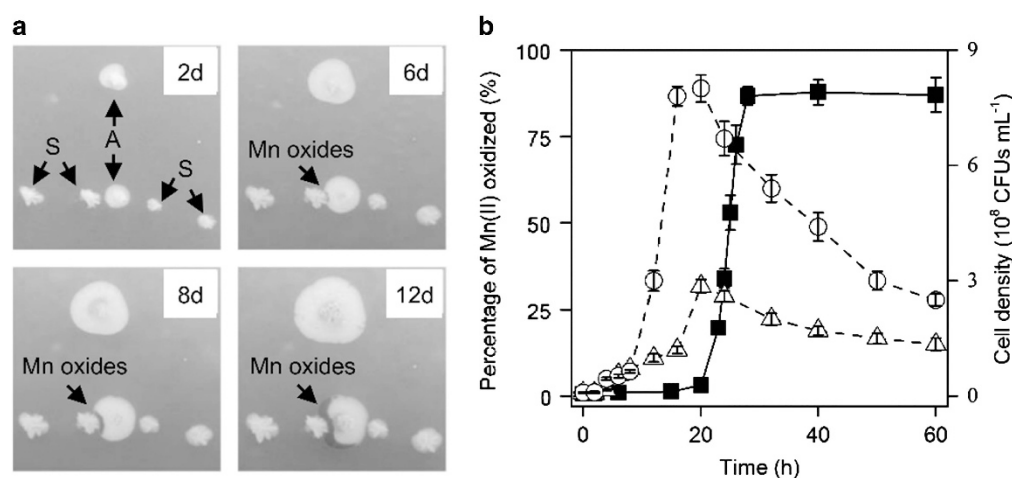

Figure 1 Mn(II) oxidation by strains *Arthrobacter* and *Sphingopyxis* in liquid media and on plate media. (**a**) Brownish Mn oxides were produced only where the colonies of *Arthrobacter* (A) and *Sphingopyxis* (S) overlapped on plate medium containing 100 μM of $MnCl_2$. (**b**) Mn(II) oxidization (squares) and colony-forming units (CFUs) (mean ± s.d., $n = 3$) of *Arthrobacter* (triangles) and *Sphingopyxis* (circles) in liquid medium, initiated with 3% (v/v) inoculum and 100 μM of $MnCl_2$.

the two strains before and after Mn(II) oxidation was visualized by scanning electron microscopy. After Mn(II) oxidation, Mn oxides were only observed on the surface of *Arthrobacter* cells, not on that of *Sphingopyxis* cells. By direct counting in the microscope field, 14 out of 64 (22%) *Arthrobacter* cells were coated with Mn oxides (Supplementary Figure S3). As Mn oxides tend to deposit onto the surface of cells that catalyze Mn(II) oxidation and *Arthrobacter* species have previously been reported to be able to oxidize Mn(II) (Ehrlich, 1971; Dubinina and Zhdanov, 1975), this indicates that Mn(II) oxidation was probably carried out by *Arthrobacter*, whose Mn(II)-oxidizing potential was inactive in isolation, and was activated in response to presence of *Sphingopyxis*.

(2) Purification and identification of Mn(II)-oxidizing protein(s). We confirmed that Mn(II) oxidation in the co-culture was enzyme-mediated, as Mn(II)-oxidizing activity in crude extracts of the co-culture

Table 1 Inhibition of Mn(II)-oxidizing activity in crude extract of co-culture cells

Treatments	Relative Mn(II)-oxidizing activity (%)
Control	100
HgCl$_2$, 10 μM	0
o-Phenanthroline, 5 μM	35
o-Phenanthroline, 25 μM	1
NaN$_3$, 100 μM	0
EDTA, 100 μM	12
60 °C, 10 min	0
Protease K, 100 μg ml^{-1}	2

Abbreviation: Mn, manganese.

cells was sensitive to a series of protein inactivation agents, as well as heat (Table 1). Co-cultures grown without Mn(II) also exhibited Mn(II)-oxidizing activities when crude cell extracts prepared using a French press were assayed. Therefore, co-culture cells grown without Mn(II) were used for Mn(II)-oxidizing protein(s) isolation to avoid interference from Mn oxides. Attempts were made to purify Mn(II)-oxidizing protein(s) from the co-culture. In the protein fractionation experiment, Mn(II)-oxidizing activity was only detected in the LBOM protein fraction. Results from native-PAGE of the LBOM protein fraction suggest that Mn(II)-oxidizing protein(s) might exhibit strong hydrophobicity. Based on the location and potential hydrophobic properties of the Mn(II)-oxidizing protein(s), we purified the Mn(II)-oxidizing proteins by combining protein fractionation with BN-PAGE. The purification method was applied to the co-culture cells (prepared by mixing 24-h pre-grown monocultures of each strain), which were collected at four time points (0, 1, 2 and 3 h after co-cultivation) to provide a visible in-gel demonstration of when the Mn(II)-oxidizing proteins were produced. Cells grown in monocultures were included as controls. For the co-culture samples, a diffused brownish Mn oxide band was observed after in-gel Mn(II) oxidation assay only for the sample at 3 h (Figure 2a, right lanes F and G). A blue protein band (Figure 2a, left lanes F and G) was also detected in the same place, sharing the identical shape with the Mn oxide band. This indicates that the proteins in the whole diffused band were all capable of Mn(II) oxidation. In contrast, for the monoculture samples after 3 h, no in-gel Mn oxide
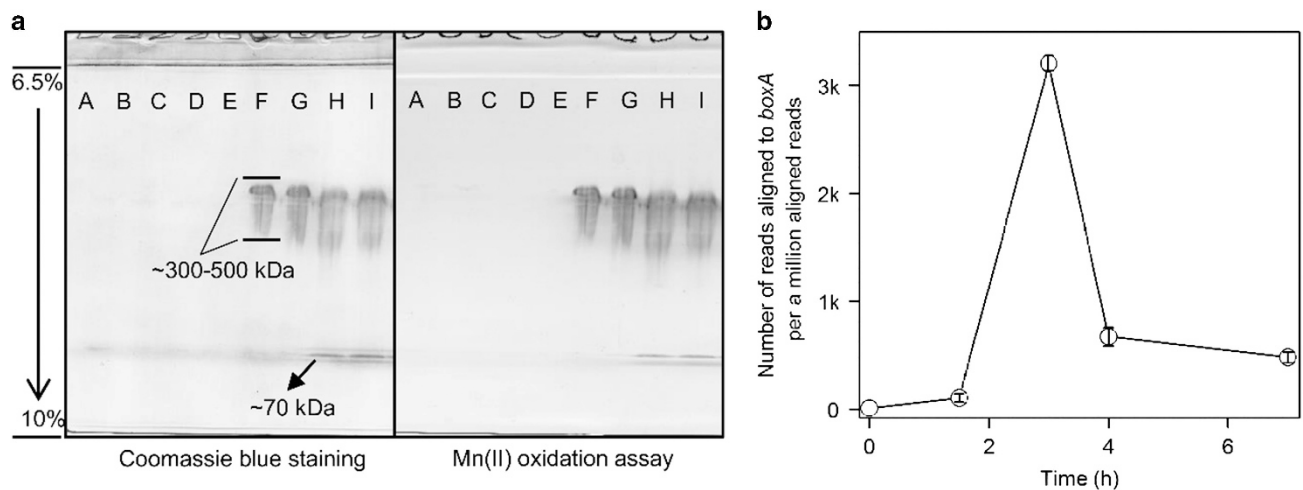

Figure 2 In-gel Mn(II)-oxidizing protein separation and abundance estimation of Mn(II)-oxidizing gene *boxA*. (**a**) Separation of LBOM proteins fraction in BN-PAGE. The two strains were cultured individually for 24 h, and then mixed for co-cultivation. Lane A, co-culture, 0 h; lane B, *Arthrobacter* monoculture, 3 h (24 + 3 h); lane C, *Sphingopyxis* monoculture, 3 h (24 + 3 h); lane D, co-culture, 1 h; lane E, co-culture, 2 h; lanes F and G, co-culture, 3 h, treated with low concentration of detergent dodecylmaltoside (DDM) (1 ×) and short equilibrium time (10 min); lanes H and I, co-culture, 3 h, treated with high concentration of DDM (2 ×) and high equilibrium time (1 h). Each well was loaded with LBOM proteins fraction extracted from 200 ml of culture cells. (**b**) Abundance estimation of Mn(II)-oxidizing gene *boxA* in co-culture, estimated from RNA-Seq data. Data are means ± average deviation for two biological replicates, except for 0 h (single sample with no replicate). The preparation of co-culture in (**b**) is identical to (**a**) except for Mn(II) addition (100 μM).

(Figure 2a, right lanes B and C) or protein band (Figure 2a, left lanes B and C) was observed at the position where the Mn(II)-oxidizing protein in the co-culture was located. These results show that the Mn(II)-oxidizing proteins were induced in the co-culture.

The proteins in the Mn(II)-oxidizing band were identified by nano LC-MS/MS. The peptides, generated by trypsin digestion, exhibited high matching score (272.35) and coverage (8.69%) (Supplementary Table S2) against a bilirubin oxidase (Accession WP_056084337; a MCO; molecular weight 74.0 kDa) in strain *Arthrobacter* sp. Leaf137. The pore size in the gel where the Mn(II)-oxidizing protein was located (Figure 2a, lanes F and G) was much larger (~300–500 kDa) than 74.0 kDa, which might relate to the aggregation of the single protein because of its strong hydrophobicity. Increasing the detergent ratio and equilibrium time in the sample preparation for BN-PAGE resulted in an additional narrow low-molecular-weight (~70 kDa) Mn(II)-oxidizing protein band in the gel (Figure 2a, lanes H and I). The proteins in the 70-kDa Mn(II)-oxidizing band were also identified by nano LC-MS/MS, and the peptides, generated by trypsin digestion, also showed a high matching score (score: 34.16; coverage: 6.63) with the bilirubin oxidase (WP_056084337) in strain *Arthrobacter* sp. Leaf137 (Supplementary Table S3). These results verified that the 70-kDa Mn(II)-oxidizing protein was identical to the ones with larger apparent molecular weights (~300–500 kDa), and was sufficient for Mn(II) oxidation. In addition, inhibition of Mn(II)-oxidizing activity in the crude extract of the co-culture cells by a metal ion chelator, phenanthroline, and a well-known MCO inhibitor, azide (Table 1), was consistent with the metalloprotein properties of MCOs. MCOs have also been verified as responsible for Mn(II)-oxidizing activity in other bacterial species (Dick *et al.*, 2008a; Butterfield *et al.*, 2013). Collectively, a Mn(II)-oxidizing protein (identified as a bilirubin oxidase) was purified from the LBOM proteins of *Arthrobacter* in co-culture, and *Arthrobacter* did not produce detectable Mn(II)-oxidizing proteins in monoculture unless grown with *Sphingopyxis*.

(3) Mn(II)-oxidizing gene. The full sequence (2040 bp) of the Mn(II)-oxidizing gene encoding the identified bilirubin oxidase in *Arthrobacter* was obtained by combining PCR with genomic walking. The nucleotide and translated protein sequences of the gene exhibited 82% and 76% similarities to the bilirubin oxidase gene sequence (locus_tag ASF64_RS17565) and the corresponding protein sequence in strain *Arthrobacter* sp. Leaf137, respectively. When the MS/MS spectra of ~ 300–500 kDa and ~ 70 kDa protein bands were searched against a database comprising all *Arthrobacter* and *Sphingopyxis* (putative) protein reference sequences and the translated protein sequence of the gene using the Proteome Discovery searching algorithm (version

1.4), credible scores (974.17 and 31.03, respectively) and coverages (49.04% and 13.40%) were observed between the MS/MS spectra and the translated protein sequence of the gene, indicating the identity between the gene and the Mn(II)-oxidizing protein. As expected, several putative copper-binding regions were present in the translated protein sequence of the gene (Supplementary Figure S4). Phylogenetic analysis (Supplementary Figure S5) revealed that protein sequence encoded by the gene exhibits distinct phylogenetic relationships with known Mn(II)-oxidizing enzymes, including CotA (Su *et al.*, 2013), MofA (Corstjens *et al.*, 1997), CueO (Su *et al.*, 2014), MoxA (Ridge *et al.*, 2007) and MnxG (Dick *et al.*, 2008a). The identified Mn(II)-oxidizing gene is therefore designated as '*boxA*'.

The transcriptional dynamics of *boxA* before and after Mn(II) oxidation in the co-culture (prepared by mixing 24-h pre-grown monocultures of each strain) were estimated from RNA-Seq data (see Supplementary Table S4). The transcript abundance of *boxA* was negligible at the beginning of co-cultivation, slightly increased at 1.5 h, and markedly increased at 3 h (Figure 2b). The time in which *boxA* was largely transcribed (3 h) was in line with the time when the Mn(II)-oxidizing protein was detected (Figures 2a and b). These results clearly indicate that *boxA* gene was silent in the *Arthrobacter* monoculture, as well as at the beginning of co-cultivation, and was activated in response to presence of *Sphingopyxis*.

(4) CFF and cell lysate experiments. In order to determine whether active *Sphingopyxis* cells are required for the induction of Mn(II)-oxidizing activity or factor(s) produced by *Sphingopyxis* is sufficient for the induction, we carried out the CFF and cell lysate experiments. Substantial Mn(II) oxidation (85%) was observed in the co-culture, whereas no
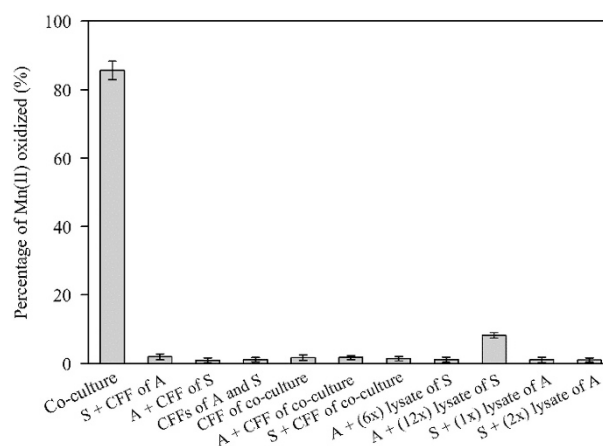

Figure 3 Mn(II) oxidation in each monoculture grown with cells, CFF, lysate of other strain and CFF of co-culture. Initial concentration of Mn(II) was 100 µM, and cultivation time was 30 h. Strain *Arthrobacter* is 'A' and *Sphingopyxis* is 'S'. Data are means ± s.d. for triplicate assays.

Mn(II) oxidation was observed in either monoculture amended with the CFF of the other monoculture or pre-incubated (24 h) co-culture (Figure 3). The results suggest that the generation of Mn(II)-oxidizing activity in the co-culture was due to the interactions between cells of one strain with cells of the other, not with the substances secreted into the medium by the other. We further examined whether cell lysate of each strain could induce Mn(II) oxidization. Interestingly, when amended with the lysate of *Sphingopyxis* cells that were 12 times as dense as *Arthrobacter* cells, *Arthrobacter* monoculture oxidized a small portion of Mn(II) (8%) (Figure 3), suggesting there is not an absolute need for *Sphingopyxis* living cells for the induction of Mn(II)-oxidizing activity of *Arthrobacter*. As expected, no Mn(II) oxidation was observed in the *Sphingopyxis* monoculture when *Arthrobacter* lysate was added (Figure 3). Taken together, the above results point toward a requirement for sustained contact between metabolically active cells of the two strains for the emergence of substantial Mn(II)-oxidizing activity.

Induction of boxA gene expression in Arthrobacter might relate to stress induced by Sphingopyxis

As substantial induction of *boxA* expression occurred at 3 h, we focused on transcripts upregulated at 1.5 h and/or 3 h. A total of 734 gene transcripts were selected accordingly (Supplementary dataset), 82.4% of which were assigned to *Arthrobacter*. We observed several upregulated transcripts with respect to stress responses (Figure 4a), including heme peroxidase transcripts (*Arthrobacter*), superoxide dismutase transcripts (*Arthrobacter*), universal stress protein transcripts (*Arthrobacter*), and chemical-damaging agent resistance protein c transcripts (*Sphingopyxis*). These data suggest generation of superoxide in the co-culture. We further detected extracellular superoxide

using superoxide-specific CL probe MCLA. The CL signal, resulting from autoxidation of MCLA probe and its reaction with superoxide, from the pre-grown (24 h) monocultures of each strain did not increase during the 30 h of extended cultivation, whereas CL signal from the co-culture (prepared by mixing 24-h pre-grown monocultures of each strain) exhibited a drastic increase during the extended cultivation, starting from 2.3 h and peaked at 3.5 h (Figure 4b). The CL signal from the co-culture decreased significantly when $50 \, kU \, l^{-1}$ superoxide dismutase was added (Figure 4b). The results indicate that extracellular superoxide was produced in the co-culture before *boxA* was substantially transcribed (3 h, Figure 2b). Considering reactive oxygen species production triggered via microbial interspecific interactions has been found in bacterial strains suffered lethal attacks from bacterial and viral species (Dong *et al.*, 2015), we postulate that *Arthrobacter* was stressed by *Sphingopyxis*, and responded by producing superoxide to counter *Sphingopyxis*, thus producing superoxide dismutase and heme peroxidase to protect itself. This was supported by the decrease of living cell densities of each strain (flow cytometry assay), as well as a faster death rate of *Sphingopyxis* than *Arthrobacter*, which occurred concomitantly with Mn(II) oxidation (Figure 4c). These results were similar to results of the CFU assay (Figure 1b), although cell ratios of the two strains differed (probably due to variations of initial cell activities). Extracellular superoxide generated by suspected *Arthrobacter* likely contributed to the faster cell death rate of *Sphingopyxis*. As enzyme-mediated Mn(II) oxidation has been shown in *Pseudomonas putida* to increase survival under oxidative stress (Banh *et al.*, 2013), *boxA* in *Arthrobacter* could then simply be another gene responding to the (oxidative) stress in the co-culture. Our data showed that Mn(II) oxidation in the co-culture might be driven by superoxide, heme peroxidase and BoxA, as the first
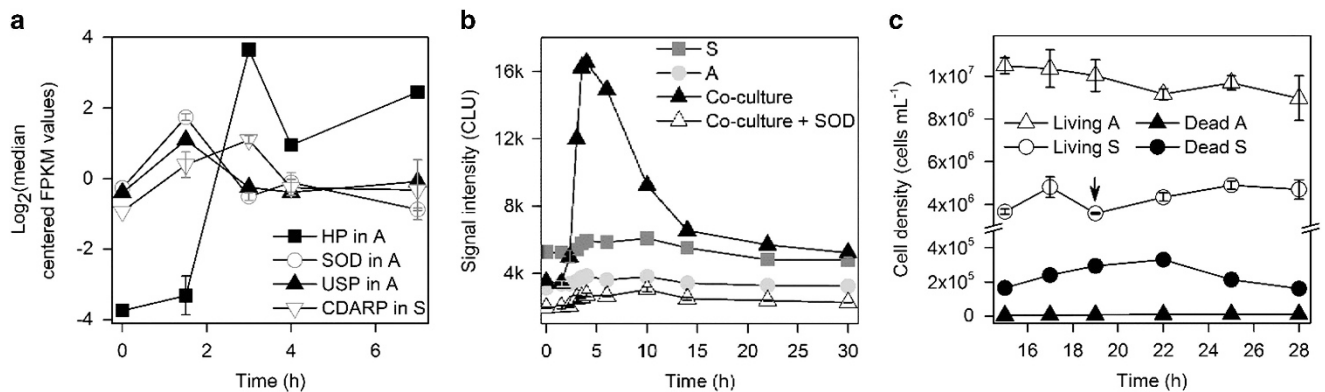

Figure 4 Stress response in the co-culture. (**a**) Abundance estimation of transcripts encoding a heme peroxidase (HP), a superoxide dismutase (SOD), and a universal stress protein (USP) in *Arthrobacter* (A) and a chemical-damaging agent resistance protein c (CDARP) in *Sphingopyxis* (S). Data are means ± average deviation for two biological replicates, except for 0 h (single sample with no replicate). (**b**) Extracellular superoxide detection in monocultures ($n = 3$) of each strain and the co-cultures (without $MnCl_2$). (**c**) Living and dead cell densities of *Arthrobacter* and *Sphingopyxis* ($n = 3$, with 100 μM $MnCl_2$) determined by flow cytometry. Arrow represents the time point when Mn oxides were detected. Note: co-culture cultivations in (**a**) and (**b**) were initiated after mixing pre-grown (24 h) monocultures of the two strains; co-culture cultivation in (**c**) was initiated with 3% (v/v) subculturing.

two products have been reported to be capable of Mn(II) oxidation (Dick *et al.*, 2008b; Learman *et al.*, 2011; Andeer *et al.*, 2015).

Our results suggest that Mn(II)-oxidizing activity in the co-culture was induced by metabolically active *Sphingopyxis* cells in a contact-dependent way, thus the stress imposed by *Sphingopyxis* on *Arthrobacter* might require cell contact. Within the upregulated transcripts (at 1.5 and/or 3 h), we further screened two contact-dependent *Sphingopyxis* transcripts, which related to bacterial type IV secretion system (T4SS) and type VI secretion system (T6SS) (Supplementary Figure S6a). Both T4SS and T6SS are widely used by Gram-negative bacteria to inject effectors into target cells (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006; Wallden *et al.*, 2010), potentially causing hydrolysis of peptidoglycan of recipient cells (Rambow-Larsen and Weiss, 2002; Russell *et al.*, 2011). Furthermore, *Sphingopyxis* transcripts encoding possible effectors (associated with infection or virulence) were upregulated (Supplementary Figure S6a). One peptidoglycan biosynthesis transcript and a phage shock protein transcript in *Arthrobacter* were upregulated, as well (Supplementary Figure S6b). These results hint at the possibility that *Sphingopyxis* injected *Arthrobacter* with effectors with peptidoglycanase activity via T4SS and/or T6SS, and induced self-defense response of *Arthrobacter*.

To summarize, we identified a novel Mn(II)-oxidizing protein and its encoding gene in *Arthrobacter*, whose expression required sustained contact with the metabolically active *Sphingopyxis* cells. Our findings highlight the roles of stress-related microbial interspecific interactions in Mn(II) oxidation and extracellular superoxide production. This study provides important insights into the contribution of biological interactions on Mn(II) oxidation in nature, and raises questions of whether microbial Mn(II) oxidation in nature is mediated by consortia, and whether our view of the diversity of Mn(II)-oxidizing bacteria is skewed by having largely identified Mn(II)-oxidizers by isolation of pure cultures.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

Author contributions

JSL, YHB and JHQ designed experiments. JSL and YHB analyzed data. Experiments were conducted by JSL. The manuscript was written by JSL and YHB, with contributions from YJM and JHQ.

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