

## Genomic Insights into Mn(II) Oxidation by the Marine Alphaproteobacterium *Aurantimonas* sp. Strain SI85-9A1<sup>∇†</sup>

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**Microbial Mn(II) oxidation has important biogeochemical consequences in marine, freshwater, and terrestrial environments, but many aspects of the physiology and biochemistry of this process remain obscure. Here, we report genomic insights into Mn(II) oxidation by the marine alphaproteobacterium *Aurantimonas* sp. strain SI85-9A1, isolated from the oxic/anoxic interface of a stratified fjord. The SI85-9A1 genome harbors the genetic potential for metabolic versatility, with genes for organoheterotrophy, methylotrophy, oxidation of sulfur and carbon monoxide, the ability to grow over a wide range of O<sub>2</sub> concentrations (including microaerobic conditions), and the complete Calvin cycle for carbon fixation. Although no growth could be detected under autotrophic conditions with Mn(II) as the sole electron donor, cultures of SI85-9A1 grown on glycerol are dramatically stimulated by addition of Mn(II), suggesting an energetic benefit from Mn(II) oxidation. A putative Mn(II) oxidase is encoded by duplicated multicopper oxidase genes that have a complex evolutionary history including multiple gene duplication, loss, and ancient horizontal transfer events. The Mn(II) oxidase was most abundant in the extracellular fraction, where it cooccurs with a putative hemolysin-type Ca<sup>2+</sup>-binding peroxidase. Regulatory elements governing the cellular response to Fe and Mn concentration were identified, and 39 targets of these regulators were detected. The putative Mn(II) oxidase genes were not among the predicted targets, indicating that regulation of Mn(II) oxidation is controlled by other factors yet to be identified. Overall, our results provide novel insights into the physiology and biochemistry of Mn(II) oxidation and reveal a genome specialized for life at the oxic/anoxic interface.**

Mn(II)-oxidizing bacteria are thought to be responsible for catalyzing the formation of most naturally occurring Mn oxides, minerals that are abundant in both marine and terrestrial environments (67). As strong adsorptive scavengers, Mn oxides control the distribution and availability of many trace elements, and as the strongest environmentally relevant oxidant after oxygen, they serve as electron acceptors for anaerobic

respiration (67). Because of the broad influence that Mn oxides exert on biogeochemical cycles, it is important to understand the biological mechanism of their formation. The ability of microorganisms to oxidize Mn(II) has been recognized for over a century, and more recently, this activity has been identified in phylogenetically diverse bacteria (70). Despite the biogeochemical and microbiological significance of Mn(II) oxidation, details of the biochemistry of this process have only recently emerged, and the physiological function of Mn(II) oxidation remains mysterious. Mn(II) oxidation by O<sub>2</sub> is thermodynamically favorable and has long been hypothesized to support chemolithoautotrophic growth, but to date, no link between autotrophy and Mn(II) oxidation has been clearly demonstrated.

The marine alphaproteobacterium *Aurantimonas* sp. strain SI85-9A1 is to date the most compelling candidate for a Mn(II)-oxidizing autotroph. Isolated from the oxic/anoxic interface (120-m depth) of Saanich Inlet in 1985 based on its ability to oxidize Mn(II) to Mn(III/IV) oxides during heterotrophic growth, it also has genes for ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (12), a key enzyme of CO<sub>2</sub> fixation by the Calvin-Benson cycle (65). These genes confer RubisCO activity to *Escherichia coli* cells in which they are heterologously expressed, but no RubisCO activity has been detected in cultures of SI85-9A1 (12). Though unable to grow with Mn(II) as the sole energy source under the laboratory

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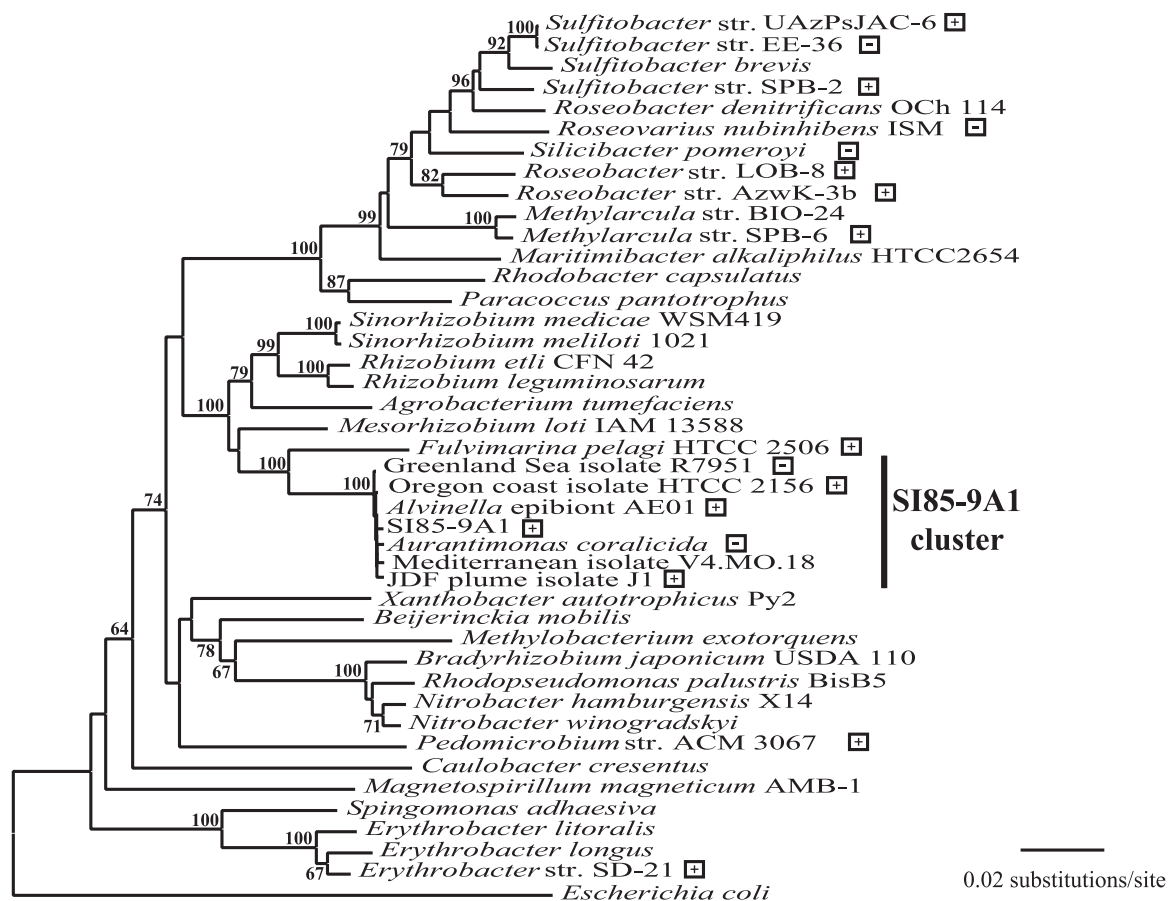


FIG. 1. Phylogeny of Mn(II)-oxidizing *Alphaproteobacteria* based on the 16S rRNA gene. Mn(II) oxidizers are denoted with "+," and isolates that have been tested and do not oxidize Mn(II) are denoted with "-." 16S rRNA gene sequences and Mn(II) oxidation data were compiled from references 21, 25, 31, 42, and 71.

conditions tested to date, SI85-9A1 has remained of interest because of the possibility that its RubisCO protein is linked to Mn(II) oxidation either under unknown conditions or during mixotrophic growth.

SI85-9A1 is one of a growing number of diverse Mn(II) oxidizers to be recognized within the *Alphaproteobacteria* (Fig. 1), where mechanisms of Mn(II) oxidation have only recently been investigated (25, 31, 42, 56). Mn(II)-oxidizing bacteria sharing >99% 16S rRNA gene nucleotide sequence with SI85-9A1 are widely distributed in diverse marine environments, from surface waters off the Oregon coast to dorsal hairs of the deep-sea hydrothermal vent tube worm *Alvinella pompejana* (Fig. 1, SI85-9A1 cluster) (21). Biochemical and genetic evidence suggests that Mn(II) oxidation by two other *Alphaproteobacteria*, strain SD-21 (25) and *Pedomicrobium* strain ACS 3067 (42, 56), is catalyzed by a multicopper oxidase (MCO) enzyme as in other Mn(II)-oxidizing bacteria (70). Still, the biochemical mechanism and physiological function of Mn(II) oxidation by *Alphaproteobacteria*, especially the SI85-9A1 group, remain largely unexplored.

In this study, we present an analysis of the genome sequence of SI85-9A1, the first report of genome-wide insights into Mn(II) oxidation. Though the focus is on Mn(II) oxidation and related processes, we briefly summarize general genome fea-

tures and overall metabolism in order to place Mn(II) oxidation into the broader context of cellular physiology. In addition to providing insights into physiological, biochemical, evolutionary, and regulatory aspects of Mn(II) oxidation, a prominent finding of this study is that the SI85-9A1 genome harbors the genetic potential to utilize diverse electron donors and carbon sources. Consistent with this view of SI85-9A1 as a metabolic generalist, we present evidence that SI85-9A1 benefits energetically from Mn(II) oxidation and suggest that this strategy might be a subtle yet environmentally relevant mode of metabolism.

#### MATERIALS AND METHODS

**Genome sequencing, annotation, and bioinformatic analysis.** DNA for genome sequencing was extracted with a Qiagen DNeasy tissue kit. Shotgun cloning and sequencing were performed by the J. Craig Venter Institute as part of the Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project (<https://research.venterlinstitute.org/moore/>). Automated annotation and complete manual curation of the SI85-9A1 genome were done with the MAGPIE system (29).

DNA binding sites for metal-sensitive transcription regulators Fur (ferric uptake regulator), Mur (manganese uptake regulator), Irr (iron-responsive repressor), RirA (rhizobial iron regulator), Iron-Rhodo, and IscR (iron-sulfur cluster regulator) were predicted using profile hidden Markov models (HMMs), generated using the hmmbuild function of software package HMMER 2.3.2 (24). Input sequences included both experimentally verified and predicted examples

TABLE 1. General genome features

Parameter	Value
Total no. of protein-coding sequences .....	3,653
No. (%) of proteins similar to those of known function .....	2,804 (77)
No. (%) of conserved hypothetical proteins.....	565 (15)
No. (%) of hypothetical proteins .....	284 (8)
No. of rRNA operons.....	3
No. of tRNA genes.....	50
% GC content.....	67

specific to *Alphaproteobacteria*, as described in supplementary Tables 1 to 7 of Rodionov et al. (58). The numbers of sequences used to construct the patterns were as follows: Irr, 221; Fur, 133; Iron-Rhodo, 126; RirA, 117; Mur, 43; IscR-1, 18; Scr-2, 7; and Mnt, 5. Trusted cutoff values were based on scores for experimentally verified examples. Putative cutoff scores were based on interspecies sequence conservation, the homology of downstream regions to genes known to be transcriptionally regulated by metal-sensitive elements, and the proximity of multiple different metal-transcription regulatory binding elements within the same upstream region.

**Phylogenetic analysis.** *moxA* homologs were identified with BLAST (minimum score of >300 and E value of <3E<sup>-83</sup>). Alignments and phylogenetic trees based on 16S rRNA gene and MoxA predicted protein sequences were constructed with a Kimura two-parameter model, using MEGA 3.1 (39). The phylogenetic methods included neighbor joining, parsimony, minimum evolution, and the unweighted-pair group method using average linkages and were tested by bootstrapping (1,000 replications). Gaps and ambiguously aligned regions were not considered.

**Growth experiments.** All growth experiments were done with J medium (68) supplemented with either methanol (0.5 to 2.5%, vol/vol), glycerol, succinate, acetate, formate, or glucose (10 mM each). Where indicated, Mn was added as MnCl<sub>2</sub> to give a final concentration of 100 μM. Optical density was measured at 600 nm. To avoid interference of Mn oxide, cultures containing Mn were reduced with 1 mM ascorbic acid prior to measurement of optical density. Oxidized Mn was quantified using the leukoberbelin blue (LBB) method (68).

**Biochemical analysis.** Cultures for biochemical analysis were grown on either J medium (supplemented with 10 mM succinate or glycerol) or M medium (68). Cultures (500 ml to 1 liter) were pelleted by centrifugation at 10,000 × g, and supernatant (1 to 4 liters) was concentrated by ultrafiltration (50-kDa molecular-mass cutoff; Millipore). Cells were lysed by passage through a French pressure cell four times at 20,000 lb/in<sup>2</sup>. Mn(II)-oxidizing activity was assayed by the LBB method (68). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) analysis and the in-gel activity assay (zymogram) were done with concentrated supernatant from stationary-phase cells grown on J-glycerol (without Mn added) as described previously (26). The zymogram was submerged in LBB to identify the presence of a Mn oxide band.

**In-gel trypsin digestion and liquid chromatography-MS/MS.** In-gel trypsin digestion of Coomassie and zymogram bands and liquid chromatography-tandem mass spectrometry (MS/MS) were performed as described previously (22). MS/MS spectra were analyzed with Analyst QS 1.1/ProID 1.1 (66) and Bio-Analyst 1.1.5/ProBlast 1.1 (60) (Applied Biosystems).

**Nucleotide sequence accession number.** Nucleotide sequence and manual annotations have been deposited in GenBank under accession number NZ\_AAPJ00000000.

## RESULTS AND DISCUSSION

**General genome features of SI85-9A1.** Shotgun sequencing of *Aurantimonas* sp. strain SI85-9A1 genome DNA resulted in 4,325,257 bp of sequence that assembled into one major contiguous sequence and 23 small fragments (41,471 bp total). A combination of automated annotation and extensive manual curation predicted 3,653 protein-coding sequences, the majority of which are most similar (at the protein level, based on BLAST scores) to genes from another recently sequenced marine bacterium, *Fulvimarina pelagi* (Tables 1 and 2). Three rRNA operons and a complete set of 50 tRNA genes were

TABLE 2. Genomes with most top BLAST hits

Organism or result	No. (%) of best hits from SI85-9A1	Total no. of genes in subject genome
<i>Fulvimarina pelagi</i> HTCC2506	1,797 (54)	3,812
<i>Mesorhizobium loti</i> MAFF303099	158 (5)	7,352
<i>Mesorhizobium</i> sp. strain BNC1	117 (4)	4,686
<i>Sinorhizobium meliloti</i> 1021	90 (3)	6,293
<i>Sinorhizobium medicae</i> WSM419	79 (2)	6,587
Other species	1,087 (33)	
No significant BLAST hits <sup>a</sup>	325 (9)	

<sup>a</sup> Significance defined as an E value of <1E<sup>-5</sup> plus >60% coverage of the query sequence by the alignment.

identified (Table 1). The genome encodes proteins for motility, including 16 predicted chemotaxis proteins and 33 predicted flagellar proteins. Mobile genetic elements are also prevalent; 73 transposons, 9 integrase/recombinases, and 11 phage-related proteins were annotated.

**Organic carbon metabolism.** Strain SI85-9A1 was isolated as a chemoorganoheterotrophic bacterium capable of oxidizing Mn(II) while growing aerobically on organic carbon. In laboratory culture, it is able to grow on a wide variety of organic carbon compounds, including organic acids (succinate, acetate, and formate), glycerol, and sugars, such as glucose. Its ability to lead an organoheterotrophic lifestyle is reflected in the genome sequence by a large number of high-affinity transporters (ABC type and TRAP [tripartite ATP-independent periplasmic transporter] type) for dicarboxylates, amino acids, peptides, and carbohydrates. An especially abundant transporter found in the SI85-9A1 genome is the predicted TRAP-type C<sub>4</sub>-dicarboxylate transport system, of which there are 19 copies. All genes required for the Embden-Meyerhof pathway (glycolysis), citric acid cycle, and pentose phosphate cycle are present, accounting for the ability to break down organic substrates and generate central precursor metabolites. Genes for key enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) were located, providing a mechanism for regenerating oxaloacetate during growth on C<sub>2</sub> or C<sub>3</sub> compounds.

**Electron transport and respiration.** A complete electron transport chain was reconstructed, including complexes I through V; therefore, all machinery for oxidative phosphorylation and aerobic respiration is present. Twenty-three genes were annotated as cytochromes, including 17 c-type cytochromes. Genes for multiple predicted terminal oxidases are present: one cytochrome *bd*-type quinol oxidase, two cytochrome *c* oxidases, and a *cbb*<sub>3</sub>-type cytochrome *c* oxidase are each encoded by clusters of three genes. In two other instances, a pair of genes annotated as cytochrome *c* oxidase subunits I and II are present, but not subunit III. The *cbb*<sub>3</sub>-type oxidase, encoded by genes *fixN*, *fixO*, and *fixP*, is predicted to have a high affinity for O<sub>2</sub>, having been found to have a K<sub>m</sub> of 7 nM in other rhizobia (54). Expression of these and other genes required for microaerobic processes is presumably mediated by the oxygen-sensitive two-component regulatory system encoded by genes *fixL* and *fixJ* (9), which are present at a separate chromosomal location. In N<sub>2</sub>-fixing rhizobia, the FixL/FixJ system also regulates the expression of genes required for nitrogen fixation, but no *nif* genes were detected in the genome of

TABLE 3. Genetic potential for metabolic versatility

Process	Protein(s)	GenBank accession no.
Putative electron donation		
Sulfur oxidation	SoxTRSVWXYZABCDEFGHI	ZP_01225759 to ZP_01225774
CO oxidation	Aerobic CO dehydrogenase	ZP_01227909 to ZP_01227911, ZP_01228739
Methylotrophy		
Methanol sensing	MoxY	ZP_01228286
Methanol regulation	MoxX	ZP_01228287
Methanol oxidation	Methanol dehydrogenase	ZP_01228289 to ZP_01228291, ZP_01228705
Formaldehyde oxidation	Formaldehyde dehydrogenase	ZP_01228291, ZP_01228274, ZP_01225718, ZP_01227698
Formate oxidation	Formate dehydrogenase	ZP_01228971, ZP_01226368, ZP_01226369, ZP_01226481, ZP_01226482
Carbon fixation		
Calvin cycle	CbbRFPTALSXE	ZP_01227925 to ZP_01227933
Calvin cycle?	RubisCO, large subunit	ZP_01226398
Unknown	RubisCO-like protein	ZP_01229072
Mn(II) oxidation		
	MCO (MoxA1)	ZP_01227197
	MCO (MoxA2)	ZP_01225555
	MCO	ZP_01225909
	MCO-like protein	ZP_01227202
	Extracellular hemolysin-type Ca <sup>2+</sup> -binding peroxidase	ZP_01225898
	Type II secretion	ZP_01225900 to ZP_01225907
	PQQ biosynthesis	ZP_01226711 to ZP_01226713

SI85-9A1, and this organism is unable to grow without supplemental fixed nitrogen. In the laboratory, SI85-9A1 is typically grown under fully aerobic conditions. Thus, SI85-9A1 appears well equipped to deal with a wide range of redox conditions, from O<sub>2</sub> saturated to microaerobic, that might be encountered in its natural habitat at an oxic/anoxic interface. The ability of SI85-9A1 to grow at low O<sub>2</sub> tension has potential significance for the biogeochemical cycling of Mn, as was recently demonstrated in the suboxic zone of the Black Sea, where rapid oxidation of Mn takes place at submicromolar O<sub>2</sub> levels (14). No evidence of anaerobic respiration was found.

**Genetic potential for utilization of inorganic and C<sub>1</sub> compounds as electron donors.** In addition to an organotrophic lifestyle, genome analysis revealed that SI85-9A1 encodes the genetic potential for utilization of several inorganic and one-carbon compounds as electron donors (Table 3). The complete suite of genes required for the oxidation of methanol to carbon dioxide is present: methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase (Table 3). Putative genes for methanol sensing and regulation were also detected. No methane monooxygenase genes were found, and key genes for the serine and ribulose monophosphate pathways of C<sub>1</sub> assimilation (phosphoenolpyruvate carboxylase and 3-hexulose phosphate synthase, respectively) were also absent. SI85-9A1 is able to grow to a limited extent with methanol as the sole energy and carbon source; therefore, carbon assimilation may occur via the energetically inefficient Calvin cycle (for which the genes are present as discussed below), as in some other facultative methylotrophs (18).

The SI85-9A1 genome contains several genes that were initially annotated as encoding the large subunit (CoxL) of carbon monoxide dehydrogenase (Table 3), the enzyme responsible for oxidizing CO to CO<sub>2</sub> in carboxydophilic bacteria

(37). Phylogenetic analysis of *coxL* showed that one of these genes clusters strongly with form II, another falls between form I and form II, and two others are only distantly related to CO dehydrogenase and are thus likely molybdenum hydroxylases with specificity for substrates other than CO (see Fig. S1 in the supplemental material). The form II *coxL* gene occurs in an operon along with genes for the small (*coxS*) and medium (*coxM*) subunits in an arrangement (*coxSLM*) that is common for form II. As in other genomes containing form II *coxL*, accessory genes (*coxBCDEFGHI*) are not present in this operon but homologs are found scattered throughout the genome (37). Although there is evidence that form II *coxL* encodes a functional CO dehydrogenase enzyme, the substrate specificity and function of these genes remain unresolved (37). The second *coxL* gene (GenBank accession no. ZP\_01228739), which falls phylogenetically between forms I and II, is present at a separate chromosomal location and without accompanying *coxM* and *comS* genes; thus, its function is unknown.

Another potential source of inorganic energy revealed through genomic analysis is reduced sulfur. The complete set of genes for sulfur oxidation is present in an arrangement (*soxTRSVWXYZABCDEFGHI*) identical to that found in the model facultative sulfur oxidizer *Paracoccus pantotrophus* (28). These genes are a faithful genetic marker of the ability of bacteria to oxidize sulfur (28); thus, their presence implies that under some conditions SI85-9A1 carries out this process. Utilization of sulfur compounds could potentially account for a substantial fraction of SI85-9A1's energy generation in its natural habitat, an oxic/anoxic interface where reduced sulfur species are abundant. Attempts to grow strain SI85-9A1 autotrophically with CO or thiosulfate as the sole electron donor have been unsuccessful so far; therefore, further physiological and biochemical work is required to determine the significance

of the *sox* and *cox* genes that we report here. One possibility is that SI85-9A1 utilizes CO and sulfur as energy supplements during heterotrophic growth, as has been recently shown to occur in other marine *Alphaproteobacteria* (48, 64).

**Autotrophy.** The complete Calvin-Benson-Bassham cycle for carbon fixation is carried in an operon of genes in the order *cbbRFPTALSXE* (Table 3). This includes genes for the key CO<sub>2</sub>-fixing enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO, encoded by *cbbL* and *cbbS*) as well as genes for regeneration of the CO<sub>2</sub> acceptor ribulose 1,5-bisphosphate: fructose-bisphosphate phosphatase (*cbbF*) and phosphoribulokinase (*cbbP*). The pentose 5-phosphate and gluconeogenesis pathways are present, allowing for the interconversion of sugar phosphates and synthesis of glucose, respectively. The presence of a gene encoding the regulator CbbR is consistent with tight control of the *cbb* operon, as in other facultative autotrophs that are metabolically versatile (40). The *cbbL* and *cbbS* genes were cloned and sequenced from SI85-9A1 previously and found to encode a functional form I, type C/D RubisCO protein when expressed in *E. coli* (12). However, no RubisCO enzyme activity has been detected in cultures of SI85-9A1 (12), and efforts to grow SI85-9A1 autotrophically on Mn(II), thiosulfate, or carbon monoxide have been unsuccessful so far. Nevertheless, the presence of the complete *cbb* operon as described here suggests that these RubisCO genes are involved in carbon fixation and are not relics of an autotrophic descendant, a possibility that was surmised previously (12). As discussed earlier (utilization of C<sub>1</sub> compounds), the Calvin-Benson-Bassham cycle could be active in carbon fixation during growth on methanol, but further experimental work is required to investigate this and other possible roles for the *cbb* genes. A second copy of *cbbL* (of unknown function) is also present, as is a predicted RubisCO-like protein (form IV) which does not function in carbon fixation but rather is thought to be involved in either sulfur metabolism, defense against reactive oxygen species, or biosynthesis (4, 32, 44).

**Mn(II) oxidation and effect of Mn(II) on growth.** Most Mn(II)-oxidizing bacteria that have been studied to date oxidize Mn(II) during late-exponential or stationary phase or upon sporulation (70). In contrast, strain SI85-9A1 oxidizes Mn(II) at an early stage of growth (Fig. 2), similar to *Pedomicrobium* sp. strain ACM 3067 (42). Mn(II) oxidation occurs during growth on organic acids (formate, succinate, and acetate) and glycerol but not during growth on glucose, despite the fact that growth is fastest on that substrate. Growth on glycerol is extremely slow but is dramatically increased upon addition of MnCl<sub>2</sub> (Fig. 2). A stimulation of growth by Mn(II) has also been observed in the Mn(II)-oxidizing alphaproteobacterium *Erythrobacter* sp. strain SD-21, but in contrast to what was found for SI85-9A1, the increase in growth was slight and occurred only at the onset of stationary phase (25). Mn is a cofactor in many enzymes and also plays a prominent biological role in protecting cells from reactive oxygen species; thus, Mn may be a growth-limiting micronutrient under certain conditions (1, 15–17, 33, 35). However, these are unlikely explanations for the Mn-stimulated growth of SI85-9A1 that we observe here. The fact that Mn(II) stimulates SI85-9A1 only during growth on glycerol, where SI85-9A1 may be energy limited, is consistent with conditions for chemolithoheterotro-

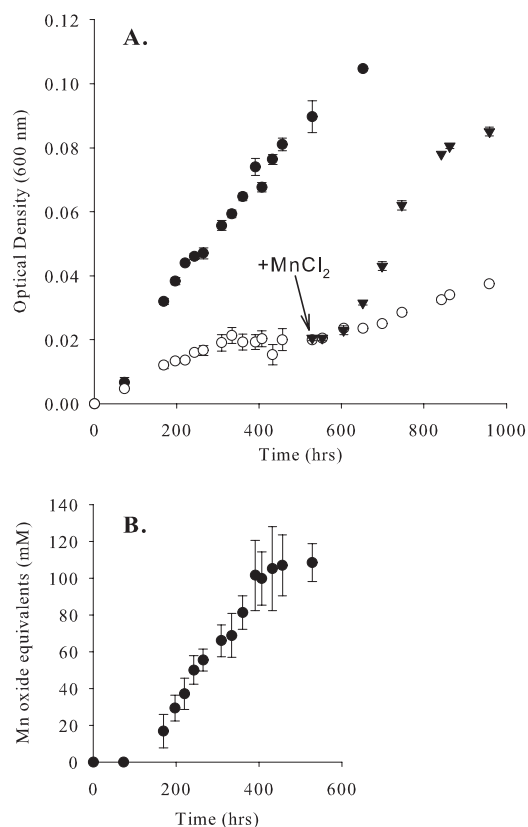


FIG. 2. (A) Growth of SI85-9A1 on glycerol with 100  $\mu\text{M}$  MnCl<sub>2</sub> (closed circles), without added Mn (open circles), and with 100  $\mu\text{M}$  MnCl<sub>2</sub> added to replicates of the no-Mn-added culture in the middle of the growth curve (closed triangles). Growth on glucose is much faster and is not stimulated by Mn(II) (data not shown; cultures reach optical densities of  $\sim 0.1$  by 200 h and  $\sim 0.8$  by 600 h). (B) Mn oxide formation by the glycerol plus MnCl<sub>2</sub> culture shown in panel A. Error bars represent standard deviations for triplicate cultures.

phy, in which inorganic compounds serve as sources of supplemental energy, allowing greater heterotrophic efficiency (3, 38, 48, 64). Mn(II) oxidation by O<sub>2</sub> is an energy-yielding reaction and could be beneficial as an energy source. Though autotrophic growth on Mn(II) has not been detected, Mn(II) oxidation could benefit the cell as an energy supplement by generating ATP or by pumping ions for gradient-driven processes, such as high-affinity transport or motility. During growth on glycerol, such a role for Mn(II) oxidation can be envisioned because ATP is required by glycerol kinase, which catalyzes the first step of glycerol utilization. Mn(II)-derived electrons could enter the electron transport chain and drive a proton gradient, consistent with cytochromes being required for Mn(II) oxidation by several Mn(II)-oxidizing species (13, 67–70). This hypothesis is supported by the presence of *c*-type cytochromes in close proximity to both copies of the putative Mn(II) oxidase (see below). Further physiology experiments are under way to investigate the nature of the stimulation of growth by Mn and to determine whether energy is derived from Mn(II) oxidation by strain SI85-9A1.

**Mechanism of Mn oxidation.** The majority of Mn(II)-oxidizing activity was recovered from growth medium supernatant of pelleted cultures rather than from whole cells or crude cell

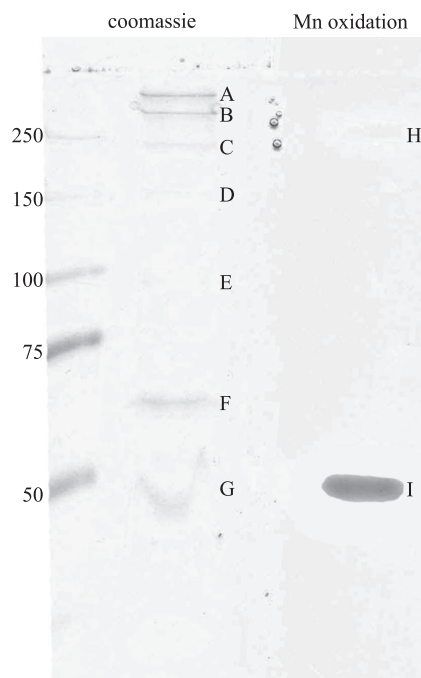


FIG. 3. SDS-PAGE analysis of the SI85-9A1 extracellular fraction during growth on glycerol. The gel was cut and one-half stained with Coomassie (left) and one-half screened with an in-gel Mn(II) oxidation activity assay (right). The latter was then immersed in LBB, which reacts specifically with Mn oxide to produce a cobalt blue color (H and I).

extracts, indicating that the Mn(II)-oxidizing enzyme is exported across the outer membrane or loosely associated with the outer membrane. SDS-PAGE analysis of concentrated cell-free growth media revealed seven distinct Coomassie-stainable bands in this extracellular fraction (Fig. 3). Six bands were unambiguously identified by mass spectrometry, whereas a seventh band contained peptides from two different proteins. Peptides from five bands (A to E) all belong to one large (predicted 350-kDa) protein that has hemolysin-type calcium-binding region signatures (Table 3) and belongs to the animal heme peroxidase superfamily (Pfam accession no. PF03098, PROSITE accession no. PDOC00394). While the role of this peroxidase is unknown, an intriguing possibility is that it may function in extracellular oxidation reactions that are related to Mn(II) oxidation. In fungi, Mn(II) serves as the reducing substrate for Mn peroxidase, which oxidizes Mn(II) to Mn(III) in the presence of organic chelators (75). Another possibility is cooperation between a Mn(II) oxidase and peroxidase, as previously reported for a litter-decaying basidiomycete: the product of Mn(II) oxidation, Mn(III), abiotically generates  $H_2O_2$ , which then serves as a substrate for Mn peroxidase in the biodegradation of lignin and xenobiotics (59). The cooccurrence of a Mn(II) oxidase (see below) and a peroxidase in the extracellular fraction of SI85-9A1 cultures suggests that a similar process may be at work. Although Mn peroxidase is best known to occur in fungi, it has also been reported to occur in bacteria (45). Two other bands in the extracellular fraction seem unrelated to Mn(II) oxidation. One band (F) was identified as a putative periplasmic substrate-binding protein from

an ABC-type transporter (GenBank accession no. ZP\_01228804), and another (G) contained peptides from two proteins, the putative periplasmic substrate-binding protein and a hypothetical protein (GenBank accession no. ZP\_01228927).

SDS-PAGE gels of the extracellular protein fraction were also screened with an in-gel activity assay (zymogram) for Mn oxidation (26), revealing one gray Mn oxide band. The composition of this band was confirmed to be Mn oxide by immersing the gel in LBB, a colorimetric indicator that reacts specifically with oxidized Mn to produce a cobalt blue color (Fig. 3) (68). This in-gel Mn oxide formation indicates a Mn(II)-oxidizing enzyme with an apparent molecular mass of ~50 kDa. The gray color is unusual as all other Mn oxide bands identified by zymogram to date are brown or orange (26); this unique color may indicate that the structure or particle size of the primary biogenic Mn oxide produced by SI85-9A1 is distinct from those of the better-studied orange/brown Mn oxides produced by *Bacillus* spores (5, 77). A second, light, LBB-positive band was apparent at >250 kDa, indicating the presence of oxidized Mn in the gel at that location as well. Since no Mn oxide band was visible at that spot prior to LBB staining, it is unclear whether this LBB band is indicative of a small quantity of Mn oxide or the presence of oxidized Mn(III) or -(IV) in some other state (e.g., bound to a protein). No peptides were recovered from either LBB-positive band; therefore, their identities are uncertain. These results are consistent with a Mn(II) oxidase being present at a very low abundance despite high Mn(II)-oxidizing activity, a characteristic observed in other Mn(II)-oxidizing bacteria (22). We are unable to discern whether the LBB-positive bands correspond to any of the Coomassie bands, because the Coomassie-stained samples shown in Fig. 3 were boiled prior to SDS-PAGE (boiling was required to get well-defined bands), but samples for the zymogram were not boiled (because boiling eliminated activity) and therefore are expected to migrate differently during electrophoresis.

All Mn(II)-oxidizing bacteria studied to date require MCO genes for Mn oxidation (70), and the MCO MnxG was recently demonstrated to be the direct catalyst of Mn(II) oxidation by spores of a marine *Bacillus* species (22). MCOs utilize Cu as a cofactor in coupling four one-electron substrate oxidations to the four-electron reduction of  $O_2$  to  $H_2O$  (63). MCOs are a large family of enzymes with high sequence divergence; however, the amino acids involved in coordinating Cu ions are highly conserved and easily recognizable. The SI85-9A1 genome contains three genes coding for proteins that contain the four Cu-binding amino acid motifs indicative of MCOs (HXH, HXH, HXXHXH, and HCHXXXH) (63), and a fourth gene encodes an MCO-like protein with three of the four MCO motifs (missing HXXHXH) (see Table 3 for GenBank accession numbers). Two of the MCOs are nearly identical to each other (95% at the amino acid [aa] level) and show significant similarity (65% aa identity) to the MoxA protein, an MCO that was found to be essential for Mn(II) oxidation in *Pedomicrobium* sp. strain ACM 3067 (56). The predicted 51-kDa sizes of the SI85-9A1 MoxA homologs (referred to hereafter as MoxA1 and MoxA2 or collectively as MoxA1/2) match the size of the Mn(II)-oxidizing enzyme identified by the Mn(II) oxidation zymogram (Fig. 3). Based on their similarity to MoxA from *Pedomicrobium* and the fact that they are predicted to be the same size as the Mn(II) oxidase, we infer that MoxA1/2 are

TABLE 4. Most closely related homologs of MoxA for which there are experimental data available

Protein	GenBank accession no.	Organism	% Identity <sup>a</sup>	Function	Source or reference
MoxA1	ZP_01227197	<i>Aurantimonas</i> strain SI85-9A1	100	Mn oxidation	This study
MoxA2	ZP_01225555	<i>Aurantimonas</i> strain SI85-9A1	98	Mn oxidation	This study
MoxA	CAJ19378	<i>Pedomicrobium</i> sp. strain ACM 3067	65	Mn oxidation	56
Orf4	AAP42069	<i>Xanthomonas campestris</i> pv. Vesicatoria	60	Cu resistance	6
PcoA	Q47452	<i>E. coli</i>	24	Cu resistance	11
Pmco	AAN52530	<i>Pseudomonas aeruginosa</i>	24	Fe(II) uptake	34
Orf1	AAA72013	<i>Xanthomonas campestris</i> pv. <i>juglandis</i>	23	Cu resistance	43
CueO	P36649	<i>E. coli</i>	23	Cu resistance	62
CopA	P12374	<i>Pseudomonas syringae</i>	23	Cu resistance	46
CopA	AAF32269	<i>Aeromonas veronii</i> bv. <i>sobria</i>	23	Slightly increased Cu tolerance	27

<sup>a</sup> Percent protein sequence identity to MoxA1.

the Mn(II)-oxidizing enzymes. In addition to being required for Mn(II) oxidation, *moxA* is required for the oxidation of the laccase substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (56). Therefore, we suspect that the putative Mn(II) oxidase(s) catalyzes the laccase-like oxidation of Mn(II) to Mn(III), but the function of the duplicate copies and their role in the oxidation of Mn(III) to Mn(IV) remain unexplained. MoxA1/2 each contain two methionine-rich regions following two of the Cu-binding regions. Methionine-rich regions have been hypothesized to play a role in binding extra Cu ions (46) that are thought to play a regulatory role in the *E. coli* MCO CueO (57).

At their N termini, MoxA1/2 have twin arginine signal peptides characteristic of the TAT secretory pathway consensus sequence (8). Whereas the TAT system targets fully folded proteins to the periplasm, it has been shown that proteins transported to the periplasm by the TAT system can be recognized and subsequently exported across the outer membrane by the type II protein secretion pathway (74), as would be expected for extracellular Mn(II)-oxidizing activity. Indeed, studies of *Pseudomonas putida* strain GB-1 have demonstrated the requirement for the type II secretion system in the transport of a Mn(II)-oxidizing enzyme, an MCO with a TAT leader sequence, across the outer membrane (10, 19). An operon encoding homologs of type II protein secretion machinery is present in SI85-9A1 (Table 3). Similar to the *P. putida* region described in the above-mentioned study, the SI85-9A1 operon is flanked by genes that are potentially involved in Mn(II) oxidation. At one end, after a transposon, is the gene for the hemolysin-type Ca<sup>2+</sup>-binding peroxidase identified by MS/MS. At the other end of the operon lies a gene for an MCO (GenBank accession no. ZP\_01225909) and a gene (GenBank accession no. ZP\_01225908) from the Sco1/SenC family of Cu chaperones that has been associated with Mn(II)-oxidizing gene regions (19, 73). The MCO in this region is most closely related to genes found in the genomes of the Mn(II)-oxidizing *Pseudomonas putida* strains GB-1 and KT2440. Although this MCO has not been experimentally implicated in Mn(II) oxidation, its genomic context and similarity to genes from distantly related Mn(II) oxidizers make it very interesting in this regard. The discovery of multiple MCOs in the SI85-9A1 genome, including two variants of the putative Mn(II) oxidase (MoxA1/2), highlights the need for a better understanding of

the exact roles of these enzymes in the biochemical mechanism of Mn(II) oxidation.

Homologs of MoxA are found in many *Alphaproteobacteria* genomes, but experimental data on the function of these genes are available for only a few distantly related MCOs (Table 4). The closest relative of MoxA (60% aa identity) is a protein (Orf4) that was recently discovered based on its involvement in Cu resistance in the plant pathogen *Xanthomonas campestris* pv. *vesicatoria* (6). The *orf4* gene is induced by Cu and is carried on the chromosome in an operon of Cu resistance genes that also share similarity with MoxA neighbors (further discussed below). Other MoxA1/2 homologs that have been experimentally associated with a function are more distantly related (Table 4). Typically, they are involved in Cu resistance, although as pointed out by Huston et al. (34), in many cases the resistance conferred is only marginal. This invites speculation that the Cu-sensitive phenotype is due to indirect effects and that the MCO may in fact have other physiological functions. Indeed, these enzymes often exhibit broad specificity, oxidizing substrates such as Fe (34), Cu (62), and organic compounds, including siderophores (30). Therefore, it has been difficult to discern whether phenotypes of MCO mutants are due to a direct role in Cu homeostasis or to loss of some other function, such as iron uptake (as demonstrated by reference 34) or siderophore oxidation.

Another area of interest with regard to Mn(II) oxidation is an operon of three genes for the biosynthesis of pyrroloquinoline quinone (PQQ), a cofactor traditionally associated with alcohol and amine dehydrogenases (2) (Table 3). This quinone has recently been found to play a role in Mn(II) oxidation by the marine alphaproteobacterium *Erythrobacter* sp. strain SD-21 (36), but the involvement of PQQ in Mn(II) oxidation by SI85-9A1 has not yet been investigated.

**mox gene context and duplication.** *moxA1* and *moxA2* occur at two separate chromosomal sites in clusters of homologous genes (Fig. 4). In both clusters, upstream of *moxA* there are genes coding for predicted proteins that are similar to MoxB (40% aa identity), also found upstream of *moxA* in *Pedomicrobium* sp. strain ACM 3067 (56). MoxB shows some similarity to the TolC family of proteins that are involved in type I secretion, and MoxB has been suggested to be involved in transport of the Mn(II) oxidase (56). However, the TolC system is also involved in the efflux of many types of molecules, including

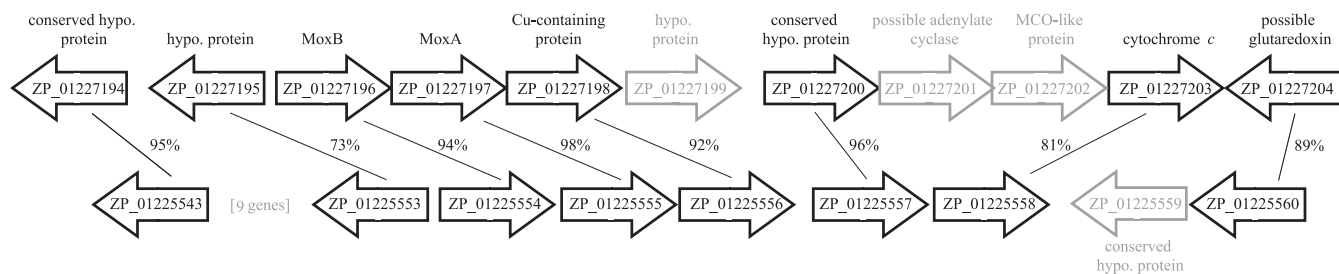


FIG. 4. Duplicated regions surrounding the *mox* genes; shown are the Mox-1 region (top) and the Mox-2 region (bottom). Predicted open reading frames are represented by arrows. Percentages indicate identity of predicted protein sequence between duplicated genes.

AQDS (anthraquinone-2,6-disulfonate), antibiotics, detergents, dyes, and phenazines (reference 61 and references therein). Further, SI85-9A1's MoxB protein also shares 31% aa identity with Orf3 from *Xanthomonas campestris* pv. vesicatoria, a protein thought to be involved in Cu resistance and to be induced by low levels of Cu (6); therefore, a number of physiological roles for MoxB seem feasible. Downstream of *moxA* is a gene encoding a small predicted protein (~18 kDa) with similarity to COG4454, an uncharacterized Cu-binding protein. This protein is similar (47% aa identity) to Orf5 of *Xanthomonas campestris* pv. vesicatoria, also thought to play a role in Cu resistance (6). The cooccurrence of *moxA*, *moxB*, and the Cu-binding-protein-encoding gene in close proximity to each other (*moxA* and *moxB* overlap by 3 bp, whereas the Cu-binding protein is 46 bp downstream in both gene clusters) in multiple species suggests that these genes are functionally related. The location of the *moxA* gene adjacent to genes that appear to be involved in Cu resistance may indicate that this putative Mn(II) oxidase also plays a role in Cu resistance. Alternatively, linkage of the MCO and the Cu homeostasis genes could merely arise from the MCO's requirement for Cu as a cofactor since these Cu resistance genes could actually be involved in Cu acquisition or sequestration.

The duplicated *mox* genes are part of larger duplicate regions (10.6 and 19.1 kb) containing genes that share significant predicted protein sequence with each other (73 to 98%) (Fig. 4). The duplicated regions share high sequence similarity, even in intergenic regions, and the Mox-1 region is flanked by transposons, suggesting that it was recently duplicated from Mox-2. Among the duplicated genes are several hypothetical and conserved hypothetical proteins as well as a *c*-type cytochrome. Cytochromes are required for Mn(II) oxidation in several Mn(II)-oxidizing bacteria (70), and it is tempting to speculate that the genetic linkage of a *c*-type cytochrome to the Mn(II)-oxidizing system reflects linkage of Mn(II)-derived electrons to the electron transport chain, as discussed above. There are also differences in gene content between the two duplicated regions, including some genes that appear to be related to Mn(II) oxidation and/or metal homeostasis (see Fig. 4 for accession numbers). In the Mox-1 region, there is an MCO-like protein that has some sequence similarity to MoxA but lacks one of the four Cu-binding regions. In the Mox-2 region upstream of *moxA*, there are nine nonduplicated genes, including a permease of the major facilitator family predicted to be a nickel resistance protein. Here, there is also a putative sensory histidine protein kinase and a two-component response regu-

lator that show some limited similarity to the CopR/PcoR and CopS/PcoS proteins, respectively, which are involved in regulating Cu resistance systems (47, 49). Genes involved in metal resistance are also present near the Mox-1 region; less than 3 kb from the region shown in Fig. 4, there is a putative heavy metal transcriptional regulator, as well as a mercury transport protein (MerT) and a mercury reductase (MerA) (GenBank accession no. ZP\_01227189 to ZP\_01227191).

**Distribution and evolutionary history of *moxA* genes.** A survey of completed and ongoing microbial sequencing genome projects revealed that *moxA*-like genes are widespread throughout the *Proteobacteria*, including the *Alpha*- (17/86 genomes), *Beta*- (21/53 genomes), *Gamma*- (1/159 genomes), and *Deltaproteobacteria* (2/19 genomes) subdivisions. Phylogenetic analysis of *moxA* and 16S rRNA gene sequences revealed stark incongruities between these two genes, indicating that *moxA* genes have been horizontally transferred in several cases (Fig. 5). The most notable examples are the *Gammaproteobacteria* *Xanthomonas campestris* pv. vesicatoria and *Pseudomonas mendocina*, whose *moxA* genes clearly cluster with the *Betaproteobacteria*, and the alphaproteobacterium *Mariaculis maris*, which has a *moxA* gene that is decidedly not of the *Alphaproteobacteria* type. This evidence for horizontal gene transfer is firm based on high bootstrap values that support the phylogeny (Fig. 5). In contrast, phylogenetic results for the clade of *Alphaproteobacteria* containing SI85-9A1 show that there are no prevalent incongruities between *moxA* and 16S rRNA genes, suggesting that *moxA* has evolved vertically in SI85-9A1 and close relatives for which genome sequences are currently available (Fig. 5). The program DarkHorse, a tool for surveying genomes for recently acquired genes (53), was used to analyze the SI85-9A1 genome. Results indicate that the number of genes in SI85-9A1 acquired through lateral transfer is relatively low, and scores for the *mox* genes were consistent with the genes having not been recently acquired from a phylogenetically distant source (see Fig. S2 in the supplemental material). Finally, percent G+C content and Karlin signatures of the *mox* regions are indistinguishable from the characteristic signatures of the overall SI85-9A1 genome, further supporting the notion that these regions have not been recently acquired. In organisms that possess multiple copies of the *moxA* gene, the two copies often cluster together (e.g., SI85-9A1), suggesting relatively recent duplication (Fig. 5B), though in a few cases, copies of *moxA* within a genome are very divergent (e.g., *Myxococcus xanthus*). The distribution of *moxA*-like genes is extremely scattered; in genera and species for which multiple



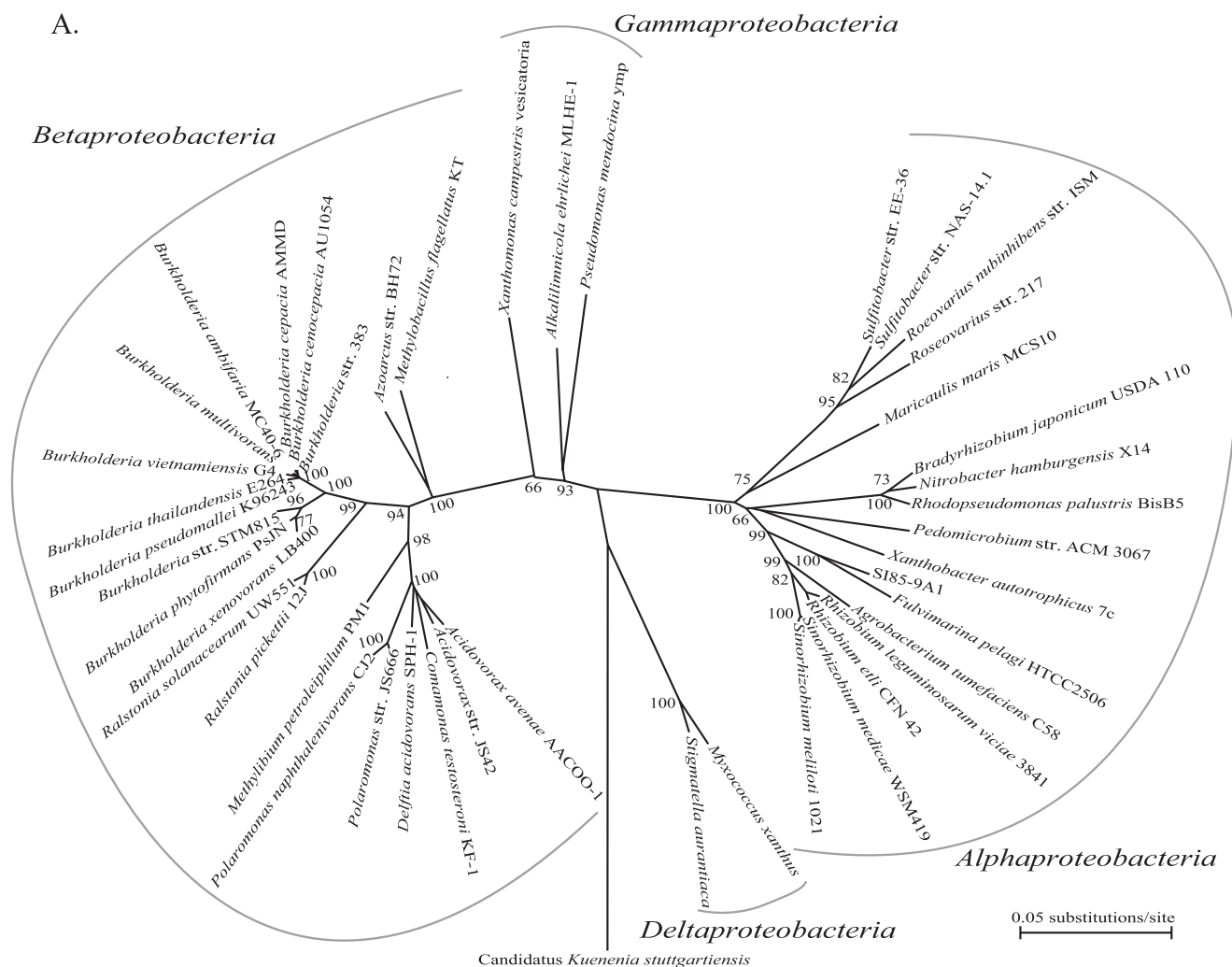


FIG. 5. Neighbor-joining phylogenetic trees based on 16S rRNA gene (A) and predicted MoxA protein sequences (B). Bootstrap values are the result of 1,000 replicates. Similar tree topologies resulted from all phylogenetic methods tested, including parsimony, minimum evolution, and the unweighted-pair group method using average linkages. In panel A, *Burkholderia pseudomallei* includes strains 1106b, 1710b, and K96243. In panel B, MoxA sequences with phylogenies apparently different from those of the 16S rRNA gene from that organism are indicated with symbols representing their 16S rRNA lineages:  $\alpha$ , *Alphaproteobacteria*;  $\gamma$ , *Gammaproteobacteria*.

genome sequences are available, such as *Burkholderia* or *Rhodopseudomonas palustris*, *moxA* is often found in some genomes but not others. Overall, these results suggest a complex evolutionary history of *moxA*, with numerous gene acquisition, loss, and duplication events. If *moxA* is indeed a genetic marker for the ability to oxidize Mn(II), this spotty distribution could explain the polyphyletic nature of Mn(II)-oxidizing bacteria (Fig. 1) (67, 68, 70).

**Metal homeostasis.** The SI85-9A1 genome sequence presents an opportunity to better understand how this organism manages the uptake, storage, and availability of Mn, Fe, and other metals. Motivation for placing Mn(II) oxidation into the broader context of cellular metal homeostasis derives from several areas of recent research. First, the putative Mn(II) oxidase is an MCO, a family of enzymes known to be involved in Fe (34) and Cu (55, 62) homeostasis and siderophore degradation (30). Second, Mn(III), an intermediate of Mn(II) oxidation (76), is bound by some siderophores with even

greater affinity than their intended target, Fe(III); thus, Mn(II) oxidation may influence Fe bioavailability (23, 51). Conversely, the formation of Mn oxides is inhibited by siderophores and thus depends on Fe bioavailability (50). Mn(II) oxidation also appears to be regulated in some cases by the concentrations of other metals, such as Zn and Pb (R. Verity and B. M. Tebo, unpublished).

SI85-9A1 has genes coding for high-affinity, siderophore-mediated ferric iron uptake systems. There are three siderophore receptors, three ABC-type siderophore transporters, and a four-gene cluster encoding siderophore biosynthesis proteins. The siderophore biosynthesis genes are most similar to those of *Rhodopseudomonas palustris* and are predicted to encode a rhizobactin-like siderophore (41). It will be interesting to determine whether this siderophore has a high affinity for Mn(III) as in other Mn(II)-oxidizing bacteria (23, 51).

The SI85-9A1 genome contains three genes of the ferric uptake regulator (Fur) family. One of these (GenBank acces-

B.

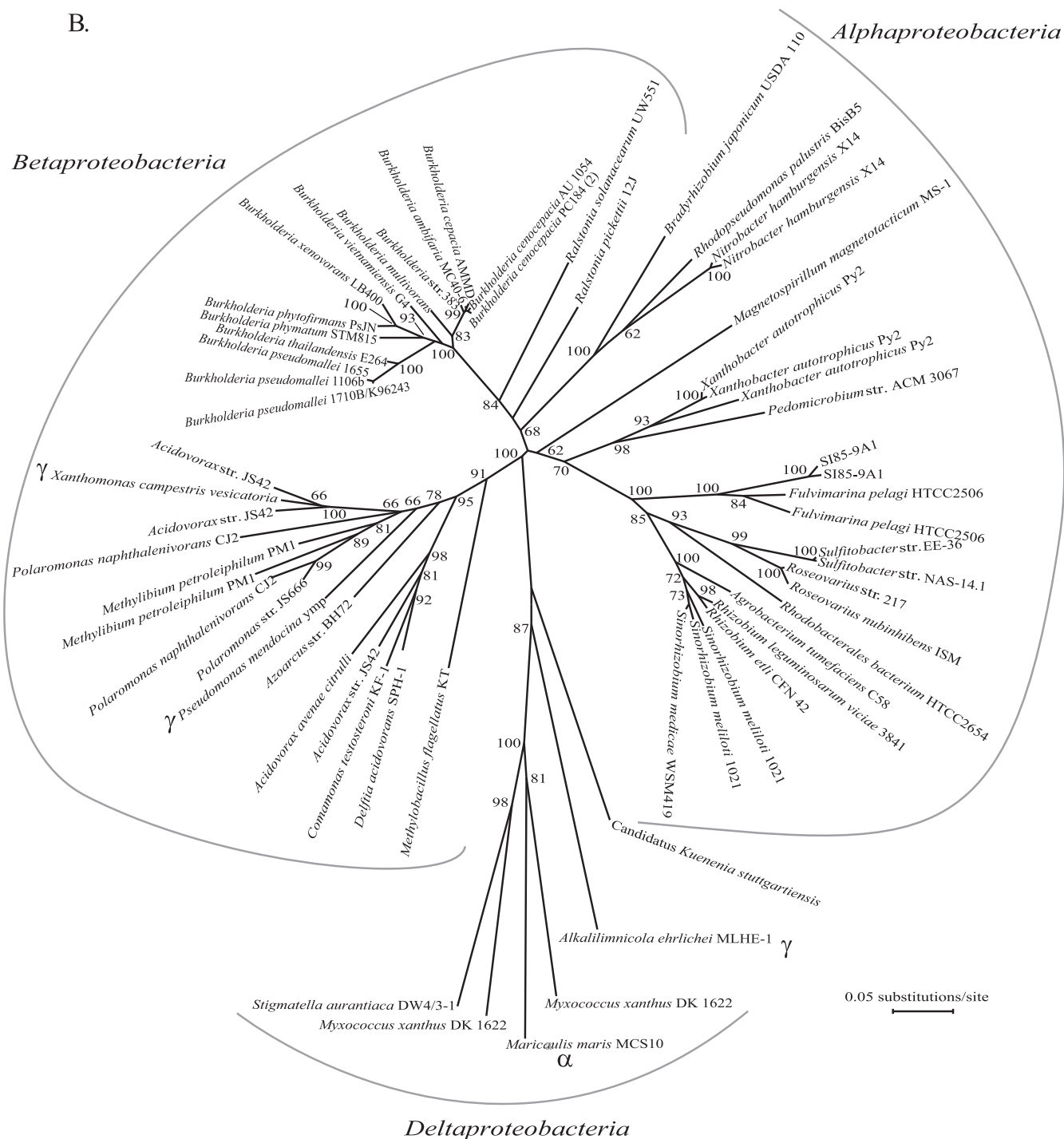


FIG. 5—Continued.

sion no. ZP\_01228946) is most similar to Fur homologs recently found to function in Mn uptake regulation (Mur) rather than Fe uptake regulation in *Alphaproteobacteria* (7, 20, 52). The major targets of this regulator, the *sitABCD* genes, encode a Mn<sup>2+</sup>-specific ABC-type transporter present in the SI85-9A1 genome (GenBank accession no. ZP\_01226419 to ZP\_01226422) and recently shown to be required for protection against oxidative stress (17). A second Fur family protein

(GenBank accession no. ZP\_01225776) is a putative Zn uptake regulator (Zur) that occurs immediately upstream of a putative ABC-type Zn transporter system, and the third Fur family protein (GenBank accession no. ZP\_01228417) clusters phylogenetically with iron response regulator (Irr) proteins that are involved in heme biosynthesis (52). SI85-9A1 also has two RirA-like proteins, additional Fe-responsive regulators that do not have any sequence similarity to Fur (72).

Robust methods for computationally identifying trace metal ion-sensitive transcription factor binding sites have recently been developed (58). Using consensus binding sequences assembled by Rodionov et al. (58), we developed an HMM to search for binding sites for transcriptional regulators Fur, Mur, Irr, Mnt, RirA, Iron-Rhodo, and IscR. Thirty-nine putative targets were identified, the majority of which have functions related to metal metabolism or are of unknown function (see Table S1 in the supplemental material), supporting the accuracy of the HMM model. Although regulation of the targets identified in the genome-wide bioinformatic search was not verified experimentally for SI85-9A1, metal-dependent regulation of homologs of many of these targets has been experimentally verified for closely related organisms (reference 58 and references therein). Among the Mur targets identified were MUR itself (GenBank accession no. ZP\_01228946) and the  $Mn^{2+}$ -specific ABC-type transporter (GenBank accession no. ZP\_01226419 to ZP\_01226422). RirA-binding sites were detected upstream of one of the CopA-type ATPases adjacent to the Mox region (GenBank accession no. ZP\_01227209) and upstream of the extracellular hemolysin-type  $Ca^{2+}$ -binding peroxidase discussed earlier. Notably, the putative Mn(II) oxidase (*mox*) genes were not among the targets predicted by our computational search of the SI85-9A1 genome. This suggests that expression of the Mn(II) oxidase is not regulated by Fe or Mn concentration, consistent with our finding that Mn(II)-oxidizing activity does not depend on the presence of added Mn(II).

Overall, the regulatory players of metal homeostasis in this marine Mn(II) oxidizer appear to be similar to those in its terrestrial rhizobial relatives *Rhizobium leguminosarum* (7, 20) and *Sinorhizobium meliloti* (52), for which the view of metal regulation is dynamic but clearly distinct from that in the better-known *Escherichia coli* and *Pseudomonas aeruginosa* systems. These  $N_2$ -fixing rhizobia require large amounts of Fe to satisfy the demands of nitrogenase; although SI85-9A1 does not have nitrogenase, apparently it retains similar mechanisms for acquiring and regulating trace metals.

**Conclusions.** The genome sequence of the alphaproteobacterium strain SI85-9A1 provides novel insights into bacterial Mn(II) oxidation and the metabolic and physiological adaptations of this organism to life in a marine oxic/anoxic interface. Previously unrecognized genetic potential for the oxidation of carbon monoxide and sulfur was revealed; along with Mn(II), these compounds represent putative sources of electrons for energy generation. Genetic markers for utilization of  $O_2$  at submicromolar concentrations suggest an ability to access such reduced compounds under redox conditions found at the oxic/anoxic interface where SI85-9A1 was isolated. Inorganic compounds, such as Mn(II), reduced sulfur species, and carbon monoxide, may provide SI85-9A1 with an energy supplement, allowing greater heterotrophic efficiency ("chemolithoheterotrophy"), as suggested for CO and sulfur in another marine alphaproteobacterium, *Silicibacter pomeroyi* (48). An additional twist on this theme is that SI85-9A1 appears, based on genome sequence, to be capable of carbon fixation via the Calvin cycle, though the electron donor and the conditions under which this occurs remain to be determined. To our knowledge, the genomic evidence of sulfur oxidation presented here is the first reported link of this ability to a Mn(II)-oxidiz-

ing bacterium. This raises some intriguing microbial biogeochemistry questions; reduced sulfur species may select for or stimulate the activities of such organisms and therefore stimulate Mn(II) oxidation rates at oxic/anoxic interfaces where Mn cycling is prevalent. Further experimental research is required to test these genome-generated hypotheses.

Two nearly identical copies of an MCO gene that putatively encodes a Mn(II) oxidase were identified. *moxA* appears to represent a new family of MCOs (56) that are widespread in isolates from diverse environments, suggesting that these genes serve important physiological and biogeochemical functions and underscoring the need for a better understanding of their function. The implication of *moxA* in Mn(II) oxidation by two phylogenetically diverse *Alphaproteobacteria*, SI85-9A1 and *Pedomicrobium* strain ACS 3067, may indicate that the MoxA-like family of enzymes are broadly capable of Mn(II) oxidation. Determination of the specificity and function of this family of enzymes requires further investigation, but an intriguing possibility is that Mn(II) oxidation is a more common trait among bacteria than is currently recognized. Overall, this study indicates that SI85-9A1 offers attractive opportunities as a model organism for furthering understanding of the physiology and biochemical mechanism of bacterial Mn(II) oxidation. In addition to the insights presented here, the genome sequence enables functional genomic approaches, such as microarrays and proteomics, that promise to address many of the hypotheses generated by this study.

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