Phylogenetic Relationships and Functional Genes: Distribution of a Gene (*mnxG*) Encoding a Putative Manganese-Oxidizing Enzyme in *Bacillus* Species[▽]

Lisa E. Mayhew,[1]* Elizabeth D. Swanner,[1] Andy P. Martin,[2] and Alexis S. Templeton[1]

University of Colorado—Boulder, Department of Geological Sciences, UCB 399, Boulder, Colorado 80309,[1] and University of Colorado—Boulder, Department of Ecology and Evolutionary Biology, Campus Box 334, Boulder, Colorado 80309[2]

Several *Bacillus* and *Paenibacillus* species were isolated from Fe and Mn oxide minerals precipitating at a deep subsurface oxic-anoxic interface at Henderson Molybdenum Mine, Empire, CO. The isolates were investigated for their Mn(II)-oxidizing potential and interrogated for possession of the *mnxG* gene, a gene that codes for a putative Mn(II)-oxidizing enzyme in *Bacillus* species. Seven of eight *Bacillus* species were capable of Mn(II) oxidation; however, the *mnxG* gene was detected in only one isolate. Using sequences of known *Bacillus* species both with and without amplifiable *mnxG* genes and Henderson Mine isolates, the 16S rRNA and *mnxG* gene phylogenies were compared to determine if 16S rRNA sequences could be used to predict the presence or absence of an amplifiable *mnxG* gene within the genomes of the isolates. We discovered a strong correspondence between 16S rRNA sequence similarity and the presence/absence of an amplifiable *mnxG* gene in the isolates. The data revealed a complex phylogenetic distribution of the *mnxG* gene in which vertical inheritance and gene loss influence the distribution of the gene among the *Bacillus* species included in this study. Comparisons of 16S rRNA and functional gene phylogenies can be used as a tool to aid in unraveling the history and dispersal of the *mnxG* gene within the *Bacillus* clade.

The comparison of 16S rRNA and functional gene phylogenies for a common set of taxa is a useful tool for attempting to understand the evolutionary history of genomes. Similarities in tree topologies are interpreted as representing the vertical inheritance of genes (23, 27, 28), while incongruities can signify the occurrence of lateral gene transfer events (14), simultaneous evolution of genes with similar functions in different organisms (28), divergence of genes from a common origin (5), or gene loss (20). If positive correlations can be made between 16S rRNA gene sequences and the presence or absence of functional genes, then 16S rRNA surveys of environmental samples can be used to understand the functional potential of the community.

The microbial oxidation of manganese (Mn) influences a number of biogeochemical processes, since Mn(III/IV) oxides are chemically reactive and can (i) sequester numerous trace metals through sorption processes, (ii) serve as terminal electron acceptors for microbial respiration, and (iii) directly oxidize complex organic matter (7, 8, 30, 33, 35). Thus, to unravel several linked chemical cycles, it is important to understand the evolution of microbial Mn(II) oxidation capabilities and the microorganisms and genes involved.

In *Bacillus* species, *mnxG*, a putative multicopper oxidase, acts to catalyze the oxidation first of Mn(II) to Mn(III) and then of Mn(III) to Mn(IV) (6, 39). *mnxG* is localized in the exosporium, a layer that occurs outside of the spore coat in some *Bacillus* species (11, 12, 38). Mn-oxidizing *Bacillus* strains have been obtained from coastal surface sediments near San Diego, CA; in sediments and particulate plumes at deep-sea hydrothermal vents in the Guaymas Basin in the Gulf of California (6, 13, 24); and now at the Henderson Molybdenum Mine in Empire, CO.

The Henderson Mine was proposed as a site for a Deep Underground Science and Engineering Laboratory (DUSEL) in 2003 and has only recently been investigated for its potential to harbor unique microbial life in the deep subsurface (29). Anoxic, CO_2- and metal-rich brines are released into aerated tunnels through boreholes at depths of >1 mile below the surface. Millimolar concentrations of Mn were detected in the fluids, and Mn oxide minerals were precipitating around the mouths of the boreholes. Eight *Bacillus* sp. isolates capable of Mn(II) oxidation were isolated from the mineral precipitates, while the *mnxG* gene was amplified from only one of these Mn-oxidizing isolates.

The inability to amplify *mnxG* from the other isolates led us to question if these isolates were more phylogenetically distinct from the *mnxG*-positive isolate than they were from each other and, if so, if 16S rRNA sequences could be used to predict the presence or absence of an amplifiable *mnxG* gene within the genomes of the Henderson Mine isolates. We hypothesized that the isolates would be dispersed throughout the *Bacillus* clade and predicted that the *mnxG*-positive isolate, HM06-02, would cluster with other *mnxG*-positive *Bacillus* isolates. We also predicted that their placement within the 16S rRNA phylogeny would relate to the presence or absence of *mnxG* within the genome of the isolates. We discovered a strong relationship between 16S rRNA sequence similarity and the presence or absence of an amplifiable *mnxG* gene in the Henderson Mine isolates. Additionally, the data revealed a complex phylogenetic distribution of the *mnxG* gene that we can use as a

* Corresponding author. Mailing address: Department of Geological Sciences, UCB 399, University of Colorado—Boulder, Boulder, Colorado 80309. Phone: (303) 492-9415. Fax: (303) 492-2606. E-mail: lisa.mayhew@colorado.edu.

TABLE 1. Organisms isolated from the Henderson molybdenum mine

Isolate	Accession no.	Sample source (depth [ft], location, type of precipitate)	Isolation medium[b]	Isolation temp (°C)	Temp (°C) optimum(a)	Mn oxidation[a]	Amplifiable mnxG	Closest relative (% identity)
HM06-01	EU004563	7,025, borehole 4	K-asw	50	Not tested	++	No	*Bacillus firmus* B563 (100)
HM06-02	EU004564	7,025, fracture, Mn oxides	K-asw	25	37	+	Yes	*Bacillus* sp. strain PL-12 (99)
HM06-02 *mnxG*	EU004573							*mnxG Bacillus* sp. strain PL-16 (98)
HM06-03	EU004565	7,025, borehole 1, Fe oxides	*Leptothrix*	50	Not tested	−	No	*Paenibacillus* sp. strain Smarlab 3301786 (99)
HM06-04	EU004566	7,025, fracture, Mn oxides	K-asw	25	37	+++	No	*Bacillus* sp. strain SD521 (99)
HM06-05	EU004567	7,025, borehole 1, Fe oxides	*Leptothrix*	50	Not tested	−	No	*Paenibacillus* sp. strain Smarlab 3301786 (99)
HM06-06	EU004568	7,025, borehole 3, Fe oxides	*Leptothrix*	50	Not detected	++	No	*Bacillus* sp. strain B-3 (99)
HM06-07	EU004569	7,025, borehole 3, Fe oxides	*Leptothrix*	50	Not tested	−	No	*Bacillus* sp. strain B-3 (100)
HM06-08	EU004570	7,025, borehole 4, Mn oxides	K-asw	50	25	++	No	*Bacillus firmus* B563 (100)
HM06-09	EU004571	7,025, borehole 4, Mn oxides	K-asw	50	Not tested	++	No	*Bacillus firmus* B563 (100)
HM06-10	EU004572	7,025, fracture, Mn oxides	K-asw	25	25 and 37	+	No	*Bacillus* sp. strain 2b-2 (98)
HM06-11	EU525854	7,025, borehole 4, Mn oxides	*Leptothrix*	50	Not tested	++	No	*Georgenia* sp. strain 3A-1 (95)

[a] +, weak; ++, medium; +++, strong; −, none.
[b] asw, artificial seawater.

tool to aid in unraveling the history and dispersal of the *mnxG* gene within the *Bacillus* clade.

MATERIALS AND METHODS

Site description and sample collection. Mn-oxidizing bacteria were isolated from deep-subsurface minerals collected from the Henderson Molybdenum Mine in Empire, CO, in March 2006. Sampling was conducted ~3,000 ft below the surface, where a series of boreholes were drilled into granite in March 2005 to release deep-seated fluids. At the time of sampling, the draining fluids were ~45°C and the ambient air temperature was ~24°C. Secondary minerals, including Fe oxides, Mn oxides, and Ca sulfates, precipitated around the mouth of each borehole. Sterile Falcon tubes were filled with mineral precipitates and stored on ice (~8 h) until return to the laboratory.

Isolation and identification of Mn(II)-oxidizing bacteria. Samples were diluted 100-fold with water from the site that had been filtered through a sterile 0.2-μm polycarbonate filter. Samples were plated onto solid medium containing organic carbon and amended with 1 mM soluble Mn(II) and incubated in the dark at 25°C or 50°C. Growth occurred on *Leptothrix* medium, a chemically defined freshwater medium for the isolation of Mn-oxidizing bacteria (25), and K medium, a seawater-based rich medium containing yeast extract and peptone as carbon sources (34) (Table 1). Single colonies were transferred until they were morphologically uniform (~6 generations), and then isolates were grown in liquid medium for DNA extraction (DNeasy blood and tissue kit; Qiagen).

PCR amplification and sequencing of 16S rRNA. The 16S rRNA gene for each isolate was amplified by PCR. The total reaction mixture volume was 50-μl: 1 μl template DNA, 50 pmol of the forward (27F, 5′-AGAGTTTGATCCTGGCTC AG-3′) and reverse (1492R, 5′-GGYTACCTTGTTACGACTT-3′) bacterial primers, 500 pmol deoxynucleoside triphosphates, reaction buffer, and 0.5 units of *Taq*. Thirty-five cycles were conducted in an Eppendorf thermal cycler (Westbury, NY) following a protocol of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, followed by 7 min at 72°C. Isolates that did not grow in liquid medium were amplified by adding a colony directly to a PCR and lysing the cells by holding them for 10 min at 94°C prior to starting the cycling routine. The PCR products were cleaned by using a QIAquick PCR purification kit (Qiagen). All sequencing was performed by SeqWright DNA Technology Services (Houston, TX). Approximately 1,400 bp of sequence was obtained by performing three sequencing reactions with 3′ primers (27F, 1074R, and 1492R) and assembling the data by using Sequencher 4.2, Gene Codes Corporation (Ann Arbor, MI). The full-

length 16S rRNA sequences were searched against the GenBank database by using BLAST (1), and the closest sequenced relatives were identified.

Determination of Mn(II)-oxidizing capabilities and amplification of *mnxG* sequences. To evaluate their Mn(II) oxidation capability, isolates were grown on plates amended with soluble Mn(II). After 1 week, the experimentally determined amount of time needed for sporulation to occur, dark colonies, potentially encrusted with brown Mn(IV) oxides, were tested for the presence of oxidized Mn by using a colorimetric leucoberbelin blue (LBB) assay (19). All *Bacillus* sp. isolates were initially tested for the presence of the *mnxG* gene by amplification with degenerate *mnxG* primers previously designed by Dick et al. (6), but only unrelated products were generated. More-specific primers, mnxGIF, 5′-ACGC ATGTCTTTCACTATCATGTTCAT-3′, and mnxGIR, 5′-AAATAAGTGGTC ATGGAAGAACCATGC-3′, previously designed by Francis et al. (13), yielded more-appropriate products. The PCR protocol was the following: 30 cycles of 94°C for 30 s, 45°C for 30 s, and 60°C for 1 min, followed by 1 cycle of 72°C for 15 min (13). PCR products were either directly sequenced by SeqWright DNA Technology Services, Houston, TX, or were cloned into chemically competent *Escherichia coli* cells by using a TOPO-TA cloning kit (Invitrogen). Clones containing the appropriate ~800-bp PCR product were purified and sequenced by using the primer M13F. A BLAST search (1) of each sequence was conducted to determine if the sequences represented an *mnxG* gene.

Phylogenetic analysis. (i) Sequence attainment. Of the 11 organisms isolated, eight were *Bacillus*, two were *Paenibacillus* sp., and one was a *Georgenia* sp. A total of 57 16S rRNA sequences were used in this study and include 24 *Bacillus* species not known to possess an *mnxG* gene, 23 sequences from *mnxG*-positive *Bacillus* organisms, the 7 sequences of *mnxG*-negative Henderson Mine *Bacillus* isolates, 1 sequence from the *mnxG*-positive Henderson Mine isolate, and the 2 sequences of the Henderson Mine isolates most similar to *Paenibacillus* species. 16S rRNA sequences were obtained by conducting nucleotide-nucleotide BLAST searches of *Bacillus* sp. strain SG-1 (1). *Bacillus* sp. strain SG-1 is a model organism used for the study of biotic manganese oxidation and possesses the *mnxG* gene (12, 24, 33, 35). The possession of an *mnxG* gene was determined by conducting a protein BLAST search using the protein sequence of the *mnxG* gene of *Bacillus* sp. strain SG-1. All *Bacillus* organisms identified in the *mnxG* search were included in the analysis to represent Mn-oxidizing members of the genus *Bacillus*. The protein product and nucleotide sequences of the *mnxG* gene in these *Bacillus* species were acquired from GenBank. BLAST searches were conducted on the 16S rRNA sequences of the isolates, and sequences of known *Bacillus* organisms with high identity to the Henderson Mine isolates (HM06-01

to -10) were included in the analysis. Many of the sequences were also used in previous Mn oxidation studies (6, 13).

(ii) Sequence alignment. 16S rRNA sequences were aligned using ClustalX (37) with default settings as follows: gap opening, 10.00; gap extension, 0.20; delay divergent sequences, 30%; DNA transition weight, 0.50; and negative matrix, OFF. An alignment of the *mnxG* protein sequence was constructed in ClustalX by using the following parameters: gap opening, 10.00; gap extension, 0.20; delay divergent sequences, 30%; protein weight matrix, Gonnet series; protein matrix, IUB; residue-specific penalties, ON; hydrophilic penalties, ON; hydrophilic residues, GPSNDQEKR; gap separation distance, 4; and end gap separation, OFF. GB02-29 was not included in the *mnxG* gene alignment because a protein sequence was not available for this organism. The nucleotide sequence of the *mnxG* gene of Henderson Mine isolate HM06-02 was translated into a protein sequence by using the translation tool ExPASy (http://www.expasy.ch/tools/dna.html) and included in the alignment.

(iii) Construction of trees. A Bayesian analysis of the aligned 16S rRNA genes from all organisms was conducted using MrBayes (17). One million generations of an MCMC run were conducted with the following parameters: nst, 6; rates, gamma; sample frequency, 100; and nchains, 4. A total of 10,000 trees were constructed; the first 1,000 trees (equal to the first 10% or 100,000 generations of the analysis) were attributed to the burn-in period and excluded from further analyses. A 50% majority consensus tree was built in PAUP (version 4.0b10) (31) by using the results of the Bayesian analysis.

PAUP (version 4.0b10) (31) was used to construct a maximum parsimony and 50% majority rule bootstrap tree by using the protein sequence of the *mnxG* gene.

(iv) Character mapping. Stochastic character mapping implemented with SIMMAP (2) allowed the estimation of the number of gains and losses of *mnxG* genes from the presence or absence data. The analysis was performed on 1,000 Bayesian 16S rRNA trees. We tested the hypothesis of no association between phylogeny and the presence or absence of *mnxG* by comparing the number of gains and losses for the observed data with the number of gains and losses after randomizing the presence or absence data across taxa 30 times, yielding a total of 30,000 mappings of gains and losses. The probability that this hypothesis is true was determined by following the method of Jones and Martin (18).

Nucleotide sequence accession numbers. The partial 16S rRNA sequences of strains HM06-01 to HM06-11 were submitted to GenBank under accession numbers EU004563 to EU004572 and EU525854 (Table 1). The single *mnxG* gene identified was submitted to GenBank under accession number EU004573.

RESULTS

Growth and Mn(II) oxidation properties of isolates. The maximum growth temperatures were determined for several isolates (Table 1). Of the 11 isolates from Henderson Mine, isolates HM06-02, -04, and -10 were grown at 25°C, while isolates HM06-05 to -09 and -11 were grown at 50°C. All isolates were examined microscopically and were rod-shaped spore formers. The results of the LBB colorimetric assay revealed that 8 of the 11 isolates exhibited Mn(II) oxidation (Table 1). Mn(II) oxidation varied from weak to intermediate to strong; this characterization was dependent on the rapidity of appearance and brightness of the blue color that indicated the oxidation of LBB by Mn(III/IV) oxides.

***mnxG* from a Henderson Mine *Bacillus* isolate is deeply nested within *mnxG* gene phylogeny.** We successfully amplified and sequenced a single *mnxG* gene from isolate HM06-02. The gene, labeled HM06-02 *mnxG*, is most closely related to sequences recovered from marine sediments from Point Loma, CA, and is deeply nested within a well-supported clade (bootstrap value = 99) of *mnxG* genes sampled from four widely different localities: Point Loma, Guaymas Basin, Mission Bay, and Henderson Mine (6, 11, 13; this work) (Fig. 1).

Phylogenetic placement of bacteria isolated from Henderson Mine. Seven unique 16S rRNA sequences were charac-
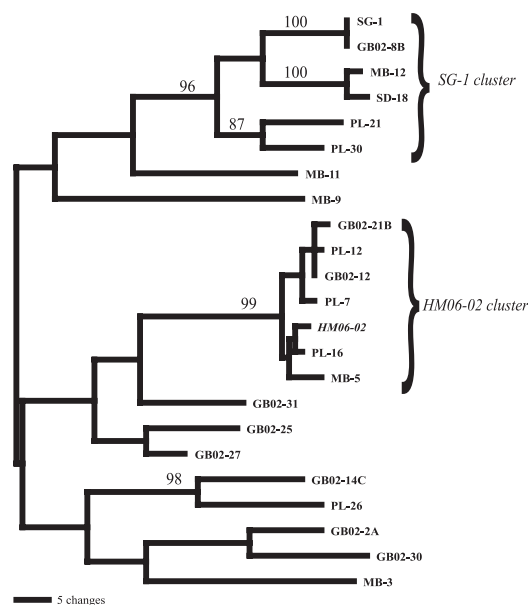

FIG. 1. Maximum parsimony bootstrap 50% majority rule consensus tree based on *mnxG* amino acid sequences. Bootstrap values are indicated at selected nodes and are based on 100 bootstrap replicates. The SG-1 cluster is labeled and is consistent across both the 16S rRNA and *mnxG* gene trees. The HM06-02 cluster is also labeled.

terized from the 11 isolates. The sequence from isolate HM06-11 was most closely identical to *Georgenia* sp. strain 3A-1, and two others (HM06-03 and -05) were identical in sequence to an isolate of *Paenibacillus* (strain Smarlab 3301786). The other sequences grouped within a clade consisting of species and isolates of *Bacillus* and *Geobacillus* (Fig. 2). The discovery of *Bacillus* species in the mineral samples was not surprising, as the cloning and sequencing of environmental DNA extracted from adjacent borehole fluids detected *Bacillus* sp. isolates with sequences 94 to 96% similar to those of the isolates in our study (29). All of the isolates from which we were unable to amplify an *mnxG* gene grouped with high posterior probabilities (posterior probability = 1.0) within clades that lacked the *mnxG* gene, whereas the one isolate possessing an amplifiable *mnxG* gene was deeply nested within a well-supported clade (posterior probability = 1.0) consisting of other species that have the *mnxG* gene (Fig. 2). The results corroborate those of our functional assays: the phylogeny of the isolated microorganisms predicted the presence and absence of the *mnxG* gene.

Few gains and frequent losses of *mnxG*. Stochastic character mapping was used to infer the history of *mnxG* gain and loss based on the presence and absence of the *mnxG* gene in taxa and the trees generated from Bayesian analysis of the 16S rRNA data. Across the many simulations of character evolution on 2,000 Bayesian trees, there were few gains of *mnxG* and many losses (Fig. 2 shows a typical example). The most-probable explanation of the data requires inferring 1 gain and 12 losses of *mnxG* (Fig. 3). A single origin of *mnxG* had the highest probability (0.566), yet the hypothesis for two and three independent gains of *mnxG* was not refuted (probabilities of 0.334 and 0.077, respectively). A broader
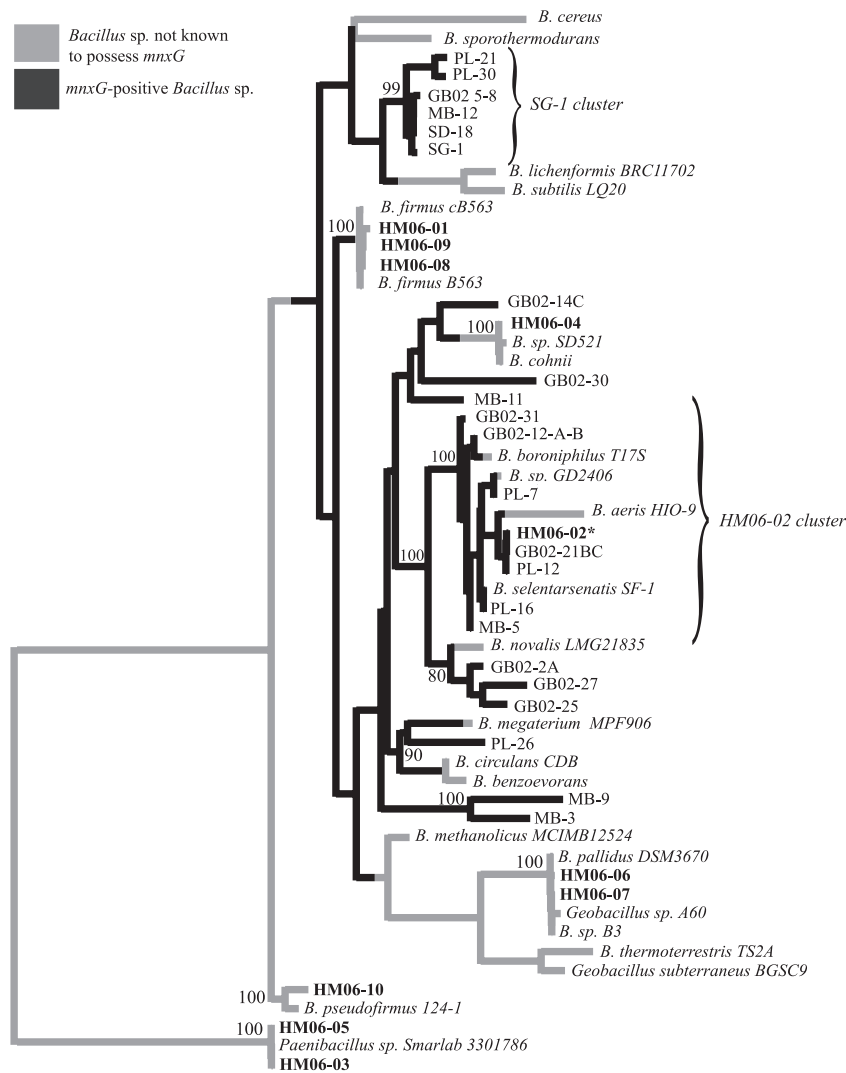
FIG. 2. Fifty-percent majority rule consensus phylogenetic tree based on 16S rRNA sequences of *Bacillus* species. Henderson Mine isolates are in boldface type, and the single asterisk indicates the *mnxG*-positive *Bacillus* organism isolated in this study. Consensus values are indicated at select nodes and are based on 9,000 Bayesian trees. The SG-1 and the HM06-02 clusters are labeled.

range of number of gene losses was observed, with most of the distribution bounded by 10 and 14 losses.

Thirteen character state changes (gains plus losses) best explained the data. This was less than expected if the character state changes were random and there was no relationship between the presence and absence of the *mnxG* gene and the 16S rRNA phylogeny of sampled isolates (Fig. 4); this indicates that vertical inheritance best explains the presence or absence of *mnxG* in *Bacillus* isolates.

DISCUSSION

Our results imply that phylogenetic placement on the basis of 16S rRNA sequence similarity predicts whether *Bacillus* species possess an *mnxG* gene. Furthermore, there is a complex pattern of distribution of the *mnxG* gene within the *Bacillus* clade revealed by the 16S rRNA relationships between
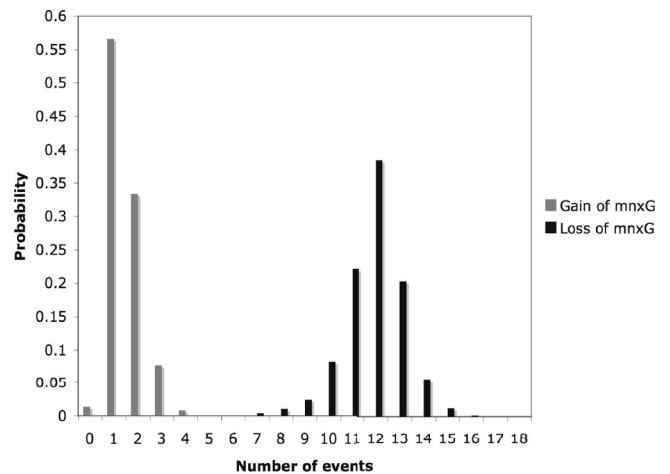

FIG. 3. Histogram depicting the probabilities of numbers of gains and losses of the *mnxG* gene needed to explain the observed data.
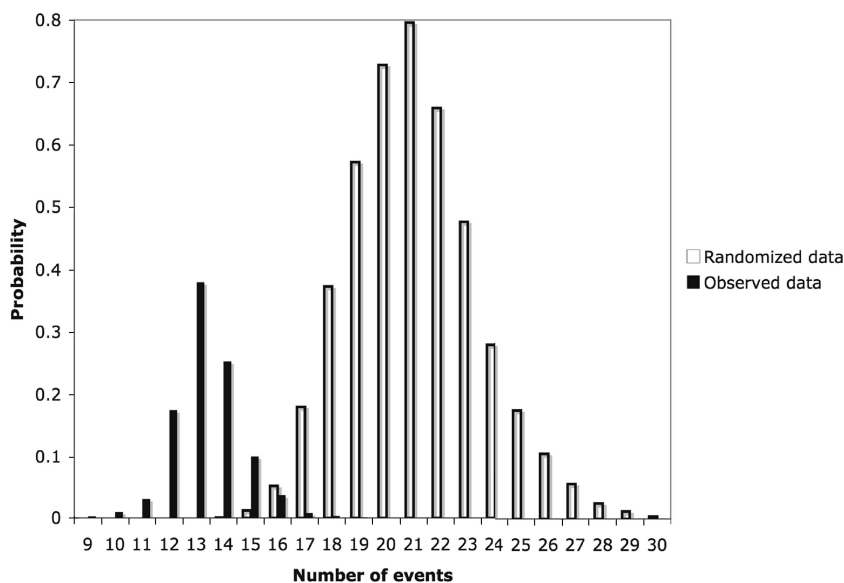
FIG. 4. Histogram comparing the number of changes needed within the trees constructed by Bayesian analysis to explain the distribution of the *mnxG* gene in the observed data versus the randomized data. Note that the distribution of observed values falls to the left of the distribution of random values; this indicates that the null hypothesis can be rejected.

the taxa. This distribution is best explained by a combination of vertical inheritance and gene loss within the *Bacillus* clade.

In the case of the Henderson Mine isolates, the results of 16S rRNA consensus tree analyses illustrate that the *mnxG*-positive isolate HM06-02 is most closely related to other *mnxG*-positive *Bacillus* organisms, while the *mnxG*-negative isolates were most closely related to other *Bacillus* organisms not known to possess an *mnxG* gene (Fig. 1). The evidence of phylogenetic conservation of the *mnxG* gene was further supported by the occurrence of an SG-1 cluster on both the 16S rRNA and *mnxG* trees (Fig. 1 and 2).

The phylogenetic inheritance of the *mnxG* gene within the *Bacillus* clade is further supported by stochastic character-mapping analyses. The most-probable number of gains and losses of the *mnxG* gene for the data set (13) is less than the number of changes (21) expected if the gene is randomly distributed on the phylogenetic trees (Fig. 4). These results indicate that phylogenetic inheritance plays a dominant role in the pattern of distribution of the gene. However, the multiple occurrences of gene loss suggest that there is a dynamic aspect to the evolution of the *mnxG* gene within the *Bacillus* clade.

The results of stochastic character-mapping analyses provided evidence for the multifaceted evolutionary process of *mnxG* within the *Bacillus* clade. Gene loss may explain relationships between taxa as seen on the 16S rRNA tree (Fig. 2), such as within the HM06-02 cluster or within the cluster of four isolates most closely related to GB02-2A, of which *Bacillus novalis* LMG21835 is the only isolate that lacks the gene (Fig. 2). The loss of genes is not uncommon and may be enhanced if the adaptation that the gene codes for is not used by the organism (4, 9, 16, 20, 22, 26). In *Bacillus* species, *mnxG* is only located in the exosporium of metabolically dormant spores (10). This may, in part, explain the frequency of loss of the gene, as the capability to oxidize Mn may not be necessary to the survival of metabolically dormant spores.

Despite the potential for the simultaneous occurrence of vertical inheritance and gene loss, it is still possible to use 16S rRNA-based phylogeny to predict to a first order the presence or absence of an amplifiable *mnxG* gene within the genome of a *Bacillus* organism.

The results of LBB tests for the presence of Mn(III/IV) oxides reveal that seven of eight Henderson Mine *Bacillus* isolates are capable of oxidizing manganese (Table 1), yet the *mnxG* gene was only amplified in one isolate, HM06-02. We present two lines of evidence supporting the observation that the *mnxG* gene is absent from the genomes of isolates HM06-01 and HM06-03 to -10: (i) the *mnxG* gene was not amplifiable, and (ii) the *mnxG*-negative organisms fall within other *Bacillus* clusters that are also not known to possess an *mnxG* gene. These data are in contrast to data from Guaymas Basin, where the authors were able to amplify an *mnxG* gene in all of their Mn(II)-oxidizing *Bacillus* species (6). There are two possible explanations for the lack of an amplifiable *mnxG* gene in Mn(II)-oxidizing *Bacillus* isolates from the Henderson Mine: (i) the *mnxG*-negative *Bacillus* sp. isolates may possess an entirely different gene that is responsible for the oxidation of manganese, or (ii) the primers designed to amplify *mnxG* may target only a subset of *mnxG* genes possessed by *Bacillus* sp. organisms. Henderson Mine *Bacillus* species are not the only examples of this paradox. *Bacillus pumilus* isolates capable of vigorously oxidizing manganese have been isolated from Mn crusts on basaltic rocks (A. S. Templeton and L. E. Mayhew et al., unpublished data), and yet *mnxG* could not be amplified from the genomic DNA in tests done in our and other labs (e.g., G. J. Dick, personal communication [regarding results of amplification using primers and conditions found in reference 6]). This was further corroborated by 16S rRNA phylogenies where *B. pumilus* does not fall within clusters of Mn(II)-oxidizing *Bacillus* species (6). Fortunately, the ge-

nome of *Bacillus pumilus* SAFR-032 has recently been completed (15). Protein sequences of all the putative manganese multicopper oxidases, *mnxG* (*Bacillus* sp. strain SG-1), *cumA* (*Pseudomonas putida* MT-2), and *mofA* (*Leptothrix discophora* SS-1), were searched against the *Bacillus pumilus* SAFR-032 genome by using BLAST. No genes homologous to *mnxG* were evident from the BLAST search; thus, it appears that *mnxG* was not amplified because it is not present in the genome. However, both *cumA* and *mofA* were most similar to *cotA* (24% and 27% identity, respectively), an outer spore coat protein of *B. pumilus*, supporting the idea that perhaps *Bacillus* sp. organisms possess multiple genes whose products have the functional capability of Mn(II) oxidation.

Conclusions. 16S rRNA phylogenies were determined to be a tool for predicting the presence or absence of a functional gene. The results of Bayesian analyses and character mapping indicate that 16S rRNA relationships between *Bacillus* organisms can be used to predict the presence or absence of the *mnxG* gene within the genome of a *Bacillus* organism. The one *mnxG*-positive Henderson Mine isolate falls in a cluster consisting predominantly of other *mnxG*-positive organisms. The *mnxG*-negative Henderson Mine isolates fall within clusters built solely of other *mnxG*-negative *Bacillus* organisms. These observations are supported by the high consensus values obtained from the Bayesian analyses. These conclusions agree with those found by other researchers investigating the relationship of 16S rRNA phylogenies to functional gene phylogenies (21, 23, 27, 28).

Gene loss was found to influence the occurrence of functional genes within the genomes of a subset of *Bacillus* species. A greater number of losses than gains have occurred (12 versus 1). In previous research, gene loss has been shown to influence the appearance of microbial genomes (4, 9). This research indicates that to fully understand the distribution of the *mnxG* gene within the genus *Bacillus*, the processes of phylogenetic inheritance and genomic remodeling through gene loss need to be considered.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
2. **Bollback, J.** 2006. SIMMAP: stochastic character mapping of discrete traits on phylogenies. BMC Bioinform. **7**:88.
3. Reference deleted.
4. **Cole, S. T., K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, K. Mungall, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. M. Davies, K. Devlin, S. Duthoy, T. Feltwell, A. Fraser, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, C. Lacroix, J. Maclean, S. Moule, L. Murphy, K. Oliver, M. A. Quail, M.-A. Rajandream, K. M. Rutherford, S. Rutter, K. Seeger, S. Simon, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, K. Taylor, S. Whitehead, J. R. Woodward, and B. G. Barrell.** 2001. Massive gene decay in the leprosy bacillus. Nature **409**:1007–1011.
5. **Dar, S. A., L. Yao, U. van Dongen, J. G. Kuenen, and G. Muyzer.** 2007. Analysis of diversity and activity of sulfate-reducing bacterial communities in sulfidogenic bioreactors using 16S rRNA and *dsrB* genes as molecular markers. Appl. Environ. Microbiol. **73**:594–604.
6. **Dick, G. J., Y. E. Lee, and B. M. Tebo.** 2006. Manganese(II)-oxidizing *Bacillus* spores in Guaymas Basin hydrothermal sediments and plumes. Appl. Environ. Microbiol. **72**:3184–3190.
7. **Dong, D., Y. M. Nelson, L. W. Lion, M. L. Shuler, and W. C. Ghiorse.** 2000. Adsorption of Pb and Cd onto metal oxides and organic material in natural surface coatings as determined by selective extractions: new evidence for the importance of Mn and Fe oxides. Water Res. **34**:427–436.
8. **Ehrlich, H. L.** 2002. Geomicrobiology, 4th ed. Marcel Dekker, Inc., New York, NY.
9. **Feldgarden, M., N. Byrd, and F. M. Cohan.** 2003. Gradual evolution in bacteria: evidence from Bacillus systematics. Microbiology **149**:3565–3573.
10. **Francis, C. A., K. L. Casciotti, and B. M. Tebo.** 2002. Localization of Mn(II)-oxidizing activity and the putative multicopper oxidase, mnxG, to the exosporium of the marine Bacillus sp. strain SG-1. Arch. Microbiol. **178**:450–456.
11. **Francis, C. A., E. M. Co, and B. M. Tebo.** 2001. Enzymatic manganese(II) oxidation by a marine alpha-proteobacterium. Appl. Environ. Microbiol. **67**:4024–4029.
12. **Francis, C. A., and B. M. Tebo.** 2001. CumA multicopper oxidase genes from diverse Mn(II)-oxidizing and non-Mn(II)-oxidizing *Pseudomonas* strains. Appl. Environ. Microbiol. **67**:4272–4278.
13. **Francis, C. A., and B. M. Tebo.** 2002. Enzymatic manganese(II) oxidation by metabolically dormant spores of diverse *Bacillus* species. Appl. Environ. Microbiol. **68**:874–880.
14. **Gersham, T. L. T., P. P. Sheridan, M. E. Watwood, Y. Fujita, and F. S. Colwell.** 2007. Design and validation of ureC-based primers for groundwater detection of urea-hydrolizing bacteria. Geomicrobiology J. **24**:353–364.
15. **Gioia, J., S. Yerrapragada, X. Qin, H. Jiang, O. C. Igboeli, D. Muzny, S. Dugan-Rocha, Y. Ding, A. Hawes, W. Liu, L. Perez, C. Kovar, H. Dinh, S. Lee, L. Nazareth, P. Blyth, M. Holder, C. Buhay, M. R. Tirumalai, Y. Liu, I. Dasgupta, L. Bokhetache, M. Fujita, F. Karouia, P. Eswara Moorthy, J. Siefert, A. Uzman, P. Buzumbo, A. Verma, H. Zwiya, B. D. McWilliams, A. Olowu, K. D. Clinkenbeard, D. Newcombe, L. Golebiewski, J. F. Petrosino, W. L. Nicholson, G. E. Fox, K. Venkateswaran, S. K. Highlander, and G. M. Weinstock.** 2007. Paradoxical DNA repair and peroxide resistance gene conservation in Bacillus pumilus SAFR-032. PLoS ONE **2**:E928.
16. **Hao, W., and G. B. Golding.** 2006. The fate of laterally transferred genes: life in the fast lane to adaptation or death. Genome Res. **16**:636–643.
17. **Huelsenbeck, J. P., and F. Ronquist.** 2001. MrBayes: Bayesian inferences of phylogeny. Bioinformatics **17**:754–755.
18. **Jones, R. T., and A. P. Martin.** 2006. Testing for differentiation of microbial communities using phylogenetic methods: accounting for uncertainty of phylogenetic inference and character state mapping. Microb. Ecol. **52**:408–417.
19. **Krumbein, W. E., and H. J. Altmann.** 1973. A new method for the detection and enumeration of manganese oxidizing and reducing microorganisms. Helgol. wiss. Meeresunters **25**:347–356.
20. **Kunin, V., and C. A. Ouzounis.** 2003. The balance of driving forces during genome evolution in prokaryotes. Genome Res. **13**:1589–1594.
21. **Kuske, C. R., S. M. Barns, C. C. Grow, L. Merrill, and J. Dunbar.** 2006. Environmental survey for four pathogenic bacteria and closely related species using phylogenetic and functional genes. J. Forensic Sci. **51**:548–558.
22. **Marri, P. R., W. Hao, and G. B. Golding.** 2006. Gene gain and gene loss in Streptococcus: is it driven by habitat? Mol. Biol. Evol. **23**:2379–2391.
23. **Martens, T., L. Gram, H. P. Grossart, D. Kessler, R. Muller, M. Simon, S. C. Wenzel, and T. Brinkhoff.** 2007. Bacteria of the Roseobacter clade show potential for secondary metabolite production. Microb. Ecol. **54**:31–42.
24. **Nealson, K. H., and J. Ford.** 1980. Surface enhancement of bacterial manganese oxidation: implications for aquatic environments. Geomicrobiology J. **2**:21–37.
25. **Nelson, Y. M., L. W. Lion, W. C. Ghiorse, and M. L. Shuler.** 1999. Production of biogenic Mn oxides by *Leptothrix discophora* SS-1 in a chemically defined growth medium and evaluation of their Pb adsorption characteristics. Appl. Environ. Microbiol. **65**:175–180.
26. **Ogata, H., S. Audic, P. Renesto-Audiffren, P. E. Fournie, V. Barbe, D. Samson, V. Roux, P. Cossart, J. Weissenbach, J. M. Claverie, and D. Raoult.** 2001. Mechanisms of evolution in Rickettsia conorii and R. prowazekii. Science **293**:2093–2098.
27. **Petri, R., and J. F. Imhoff.** 2000. The relationship of nitrate-reducing bacteria on the basis of *narH* gene sequences and comparison of *narH* and 16S rDNA-based phylogeny. Syst. Appl. Microbiol. **23**:47–57.
28. **Petri, R., L. Podgorsek, and J. F. Imhoff.** 2001. Phylogeny and distribution of the soxB gene among thiosulfate-oxidizing bacteria. FEMS Microbiol. Lett. **197**:171–178.
29. **Sahl, J. W., R. Schmidt, E. D. Swanner, K. W. Mandernack, A. S. Templeton, T. L. Kieft, R. L. Smith, W. E. Sanford, R. L. Callaghan, J. B. Mitton, and J. R. Spear.** 2008. Subsurface microbial diversity in deep-granitic-fracture water in Colorado. Appl. Environ. Microbiol. **74**:143–152.
30. **Sunda, W. G., and D. J. Kieber.** 1994. Oxidation of humic substances by

manganese oxides yields low-molecular weight organic substrates. Nature **367:**62–64.

31. **Swofford, D. L.** 2000. PAUP*: phylogenetic analysis using parsimony (* and other methods). Sinauer Associates, Sunderland, MA.
32. Reference deleted.
33. **Tebo, B. M., J. R. Bargar, B. G. Clement, G. J. Dick, K. J. Murray, D. Parker, R. Verity, and S. M. Webb.** 2004. Biogenic manganese oxides: properties and mechanisms of formation. Ann. Rev. Earth Planetary Sci. **32:**287–328.
34. **Tebo, B. M., B. G. Clement, and G. J. Dick.** 2007. Biotransformations of manganese, p. 1223–1238. *In* C. J. Hurst, R. L. Crawford, J. L. Garland, D. A. Lipson, A. L. Mills, and L. D. Stetzenbach (ed.), Manual of environmental microbiology, 3rd ed. ASM Press, Washington, DC.
35. **Tebo, B. M., H. A. Johnson, J. K. McCarthy, and A. S. Templeton.** 2005.

Geomicrobiology of manganese(II) oxidation. Trends Microbiol. **13:**421–428.
36. Reference deleted.
37. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The ClustalX-Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
38. **Van Waasbergen, L. G., M. Hildebrand, and B. M. Tebo.** 1996. Identification and characterization of a gene cluster involved in manganese oxidation by spores of the marine *Bacillus* sp. strain SG-1. J. Bacteriol. **178:**3517–3530.
39. **Webb, S. M., B. M. Tebo, and J. R. Bargar.** 2005. Structural characterization of biogenic Mn oxides produced in seawater by the marine Bacillus sp. strain SG-1. Am. Mineral. **90:**1342–1357.
40. Reference deleted.