http://www.aimspress.com/journal/environmental

AIMS Environmental Science, 3(2): 220-238.
DOI: 10.3934/environsci.2016.2.220
Received 21 January 2016,
Accepted 05 April 2016,
Published 12 April 2016

Research article

Manganese-II oxidation and Copper-II resistance in endospore forming Firmicutes isolated from uncontaminated environmental sites

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Abstract: The accumulation of metals in natural environments is a growing concern of modern societies since they constitute persistent, non-degradable contaminants. Microorganisms are involved in redox processes and participate to the biogeochemical cycling of metals. Some endospore-forming Firmicutes (EFF) are known to oxidize and reduce specific metals and have been isolated from metal-contaminated sites. However, whether EFF isolated from uncontaminated sites have the same capabilities has not been thoroughly studied. In this study, we measured manganese oxidation and copper resistance of aerobic EFF from uncontaminated sites. For the purposes of this study we have sampled 22 natural habitats and isolated 109 EFF strains. Manganese oxidation and copper resistance were evaluated by growth tests as well as by molecular biology. Overall, manganese oxidation and tolerance to over 2 mM copper was widespread among the isolates (more than 44% of the isolates exhibited Mn (II)-oxidizing activity through visible Birnessite formation and 9.1% tolerate over 2 mM copper). The co-occurrence of these properties in the isolates was also studied. Manganese oxidation and tolerance to copper were not consistently found among phylogenetically related isolates. Additional analysis correlating the physicochemical parameters measured on the sampling sites and the metabolic capabilities of the isolates showed a positive correlation between *in situ* alkaline conditions and the ability of the strains to perform manganese oxidation. Likewise, a negative correlation between temperature in the habitat and copper tolerance of the strains was observed. Our results lead to the conclusion that metal tolerance is a

wide spread phenomenon in unrelated aerobic EFF from natural uncontaminated environments.

Keywords: Endospore-forming Firmicutes; Cooper resistance; Manganese oxidation; metal contamination; natural habitats

1. Introduction

The release of metals from various industries [1,2], raises particular concern because they constitute persistent environmental contaminants that cannot be degraded or destroyed. At high concentrations, metals are toxic to living cells and accumulate throughout the food chain, leading to serious ecological and health issues [3]. Despite their toxicity, living organisms require some specific metals (e.g. Ni, Cu, Co, Cr, Fe, Ca, Zn, K, Mn, and Mg) at low concentrations as micronutrients and they play a vital role in metalloenzymes as cofactors [4].

Environmental contamination by metals can have a significant impact on the indigenous microbial populations. Nearly every index of microbial metabolic activity (respiration, methanogenesis and nitrogen fixation, among others) is adversely affected by elevated concentrations of toxic metals [5]. Consequently, microorganisms that thrive in metal-contaminated environments have developed a variety of strategies for their survival including detoxifying mechanisms such as bioaccumulation, biotransformation, biomineralization or biosorption. Those mechanisms are of interest for *ex situ* or *in situ* bioremediation technologies [3,6-9].

Manganese (II) oxidation is of particular relevance in environmental studies because Mn oxides are among the strongest naturally occurring oxidizing agents in the environment and can play a role in numerous redox reactions controlling the distribution of other trace and contaminant elements [10]. Despite the evidence indicating a large phylogenetic diversity among bacteria able to oxidize Mn (II), the evolutionary origin and function of this metabolic process are still under debate [10]. One hypothesis considers the possibility that by coating themselves with Mn oxides, Mn (II)-oxidizing bacteria create a self-made protection layer from environmental insults such as UV radiation, predation, viral attack or even metal toxicity [10]. The latter possibility would suggest that Mn (II)-oxidization could be a good predictor of the ability of an organism to resist elevated concentrations of toxic metals. However, this has not been tested experimentally.

An intriguing physiological link might exist between manganese oxidation and copper tolerance. One of the enzymes identified so far as having an integral role in Mn (II)-oxidation are multicopper oxidases (MCOs) [11,12]. These enzymes participate in a variety of cellular functions including copper homeostasis [13,14]. Copper homeostasis is a complex process mediated by various genetic determinants [1,2], involving sequestration, uptake, and efflux [13]. Hence, Mn (II) oxidation and copper tolerance/resistance may be interrelated phenomena, but this has not been previously studied.

In terms of the diversity of microorganisms able to tolerate metals, tolerance has been mainly investigated in bacteria that are constantly under the selective pressure of high metal contents. This explains why, in environments enriched with specific metals, metal-resistant bacteria are predominant [15-18]. In these communities, endospore-forming Firmicutes (mainly Bacilli and Clostridia) are reported as a major portion of the total bacterial communities [15,19,20]. In the case of endospore-forming Firmicutes (EFF), their endospores are resistant to physical and chemical shock,

enabling persistence under stressing conditions for many years without losing germination potential [21]. In addition to their high prevalence in contaminated sites and their survival capability, EFF are known to participate in the biogeochemical cycling of metals, facilitating oxidation/reduction processes [11,22]. For instance, *Bacillus* sp. strain SG1 and *Desulfotomaculum reducens* strain MI-1 have been involved in manganese (II) oxidation [23] and uranium (VI) reduction [24], respectively. The widespread ability to oxidize manganese found among aerobic endospore-forming Firmicutes makes this group an ideal candidate to study the hypothesis of a correlation between Mn (II)-oxidation and tolerance to toxic metals, and more specifically to copper. In this study, a series of aerobic endospore-forming Firmicutes were isolated from a diverse set of natural environments and their capability to oxidize Mn (II) was evaluated experimentally. In parallel, tolerance to increasing concentrations of copper was measured. The results were analyzed in order to establish a link between both metabolic process and the phylogenetic identity of the strains.

2. Materials and Method

2.1. Site description and sample collection

Various environmental samples such as soil, water, sediments and biofilms were collected from Chile (CL), Colombia (CO), Greece (GR), France (FR), Germany (DE) and Switzerland (CH) from March 2010 to April 2012. The samples were collected in sterile 50-ml Falcon tubes and transported to the laboratory on ice and stored at 4 °C for bacterial enrichment and isolation. The sampling sites corresponded to environmental niches that have not been contaminated by industrial deposition of metals. These environments and their geochemical characteristics are summarized in Supplementary Table S1.

2.2. Enrichment and isolation

For enrichment, one gram of collected sample was inoculated into 10 mL of Nutrient Broth (Biolife) and 10 mL of modified DifcoTM Marine Broth 2216 (D2216*, 5 g of tryptone instead of peptone, no potassium bromide, pH adjusted 5.5, 6.8, 7.4 with 2N HCl). The inoculated media were incubated at specific temperatures corresponding to those of the sampling sites under aerobic conditions for 72 h. The enriched cultures were then plated on Nutrient Agar (NA) and modified Marine Agar (MA) (modified Marine Broth with 2% agar). Single colonies were obtained and each colony was plated repeatedly to attain pure aerobic bacterial isolates. Pure cultures were stored at 4 °C and cryopreserved in 25% (v/v) glycerol at −80 °C. Morphological and colony features were described according to the minimal standards for describing new taxa of aerobic endospore-forming bacteria [25]. Colony morphology was observed after 12 h of growth. Cell morphology, average cell size at 24 h in liquid medium and endospore formation were determined using phase-contrast microscopy (Leica DM R, magnification 1000x). EFF were selected among other isolates based on their capability to form spores after starvation for 15 days.

2.3. Identification of the isolated strains

Genomic DNA from each strain was extracted using the InnuPREP Bacteria DNA kit (Analytik

Jena, Germany), according to the manufacturer's instructions. For amplification of 16S rRNA gene, the primers GM3F, GM4R and Eub9_27, Eub1542 were used as previously described [26,27]. Presence of the gene that encodes the transcriptional factor responsible for the initiation of sporulation in endospore-forming bacteria (*spo0A* gene) was verified with the specific set of primers spo0A166f and spo0A748r, as described previously [28]. The PCR products were purified with a MultiScreen PCRµ96 Filter Plate (Millipore, USA), according to the manufacturer's instructions and sequenced using the services of Microsynth AG (Switzerland) and GATC Biotech (Germany). Nearly full-length 16S rRNA sequences were obtained by sequencing the PCR products in addition with the primers 907r, 926f primers and 518r [27,29]. The 16S rRNA genes were identified using the services of EzTaxon, against EzTaxon's cultured isolates database [30]. The sequences were submitted to GenBank under accession numbers KJ722422-KJ722533.

2.4. Manganese (II)-oxidation

Aerobic endospore-forming Firmicutes were screened for their capability to perform Mn (II) oxidation. Mn (II) oxidation was initially detected by the formation of brown Mn oxides on sporulated colonies after 10 days of incubation on solid K medium plates [22]. Mn (II) oxidation was further confirmed by Birnessite formation due to the oxidation of Mn (II) to crystalline Mn (IV) oxides in *Leptothrix* liquid medium [31]. Endospore-forming Firmicutes were also screened for the presence of a Mn oxidation gene that encodes a multicopper oxidase (MCOs) also responsible for copper resistance (*mnxG*), using primers adapted for *Bacillus* spp. [22]. The PCR products were purified on a MultiScreen PCRµ96 Filter Plate (Millipore, USA), according to the manufacturer's instructions and sequenced in order to confirm the identity of the PCR products as *mnxG* gene. Sequencing was performed using the services of GATC Biotech (Germany).

2.5. Copper (II) resistant/tolerance

The screening of Cu (II) resistant strains was carried out by using a modified form of Nutrient Agar and DifcoTM Marine Agar 2216* amended with 0.5 mM of $CuSO_4 \cdot 5H_2O$ (II) (Sigma). The bacterial strains were inoculated onto agar plates and were incubated at each isolate's optimal temperature for 3–5 days. The level of Cu (II) resistance of the bacterial strains, that already grew at 0.5 mM Cu (II), was determined by plating the colonies on agar plates with increasing concentrations of $CuSO_4 \cdot 5H_2O$ (II) from 0.75, 1.0, 1.5 up to 3.0 mM. Further, high Cu (II) tolerant strains were selected to test their Minimum Inhibitory Concentration (MIC). MIC was determined in nutrient or marine broth amended with 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 to 8 mM Cu (II). Strains were incubated at optimal temperatures for at least 1 week, verifying bacterial growth by optical density at 600 nm every 24 h. MIC was estimated as the first dilution which completely inhibits bacterial growth in nutrient or marine medium. The initial and final concentration of Cu (II) in the MIC cultures was quantitatively measured using a spectrophotometric copper assay [32]. The final pH was also measured at the end of the incubation.

2.6. Statistical Analysis

Statistical analyses were performed using Rstudio, version 0.98.1049. Correlations between diversity and environmental limiting factors were estimated using both Pearson's and Spearman's

methods, however since our data are not normally distributed and also taken or transformed into ordinal scale, Spearman's tests were considered as more appropriate and therefore applied to this dataset.

2.7. Phylogenetic analysis of 16S rRNA, Mn (II) oxidation and Cu (II) resistance

Sequences of the 16S rRNA gene corresponding to the 109 strains analyzed were aligned using MAFFT [33,34]. A maximum likelihood phylogenetic tree was built using PhyML [35] with default parameters, and then an ornament tree was created using Newick utilities [36].

3. Results

3.1. Isolation and identification of endospore-forming Firmicutes

Aerobic endospore-forming Firmicutes of 22 non-polluted natural environmental sites from six countries (CL, DE, FR, CO, CH, GR) were characterized. A total of 338 strains representing distinct morphologies (including two phase variants) were selected from the different selective conditions and purified on NA and MA agar plates. All presumptive endospore-formers were selected based on their sporulating activity, the presence of *spo0A* gene and their 16S rRNA gene sequence. Those corresponded to 109 endospore-forming strains (Table 1).

Table 1. Genera isolated per sampling site and number of isolates per genus.

Sampling Site	Genera	Number of Isolates
Aggistro (GR)	*Bacillus*	1
(water and soil from geothermal spring)	*Lysinibacillus*	1
Agia Paraskevi (GR)	*Exiguobacterium*	4
(water and soil from underground		
geothermal sulfuric spring)	*Sporosarcina*	1
Aguas Calientes (CL)		
(inlets of thermal water, salt crusts)	*Bacillus*	1
Bruschal (DE)	*Anoxybacillus*	7
(high pressure high temperature	*Aeribacillus*	1
oligotrophic geothermal reservoir)	*Brevibacillus*	2
El Tatio (CL)	*Anoxybacillus*	2
(geothermal geysers, hot pools,	*Bacillus*	8
hot streams and precipitates)	*Geobacillus*	3
Eleftheres (GR)		
(water and soil from geothermal spring)	*Bacillus*	1
Huasco (CL)		
(white sediment with salt precipitation from thermal salar)	*Bacillus*	1
Krinides (GR)	*Bacillus*	2
(water and biofilm from geothermal spring)	*Kurthia*	1
Lagadas (GR)		
(water and biofilm from geothermal spring)	*Bacillus*	1

Las Piedras (CL) (sediment from salars)	*Bacillus*	4
Lirima (CL) (microbial mats and sediment from thermal sulfur springs and streams)	*Anoxybacillus* *Bacillus* *Lysinibacillus*	8 13 1
Milos (GR) (soil from volcanic crater, water from hydrothermal springs, biofilm from geothermal springs and drillings)	*Aeribacillus* *Bacillus* *Geobacillus*	3 9 1
Nea Apollonia (GR) (water and biofilm from geothermal spring)	*Bacillus*	5
Neuchatel (CH) (water and soil from mineral sulfur and iron springs)	*Bacillus* *Exiguobacterium* *Lysinibacillus*	2 2 1
Nigrita (GR) (water and biofilm from geothermal spring)	*Aneurinibacillus* *Bacillus*	2 5
Pikrolimni (GR) (water and sediment from thermal lake)	*Bacillus*	2
Potamia (GR) (water and biofilm from geothermal spring)	*Anoxybacillus*	1
Pozar (GR) (water and biofilm from geothermal spring)	*Bacillus* *Lysinibacillus*	2 1
Soulz-sous-forets (FR) (high pressure high temperature oligotrophic geothermal reservoir)	*Bacillus* *Geobacillus*	3 1
Thermia (GR) (water and biofilm from geothermal spring)	*Anoxybacillus* *Geobacillus*	1 2
Traianoupoli (GR) (water and biofilm from geothermal spring)	*Bacillus*	2
Yungai (CL) (sand and salt crusts)	*Bacillus*	2

3.2. Mn (II)-oxidation

Two types of screening medium (K medium and Birnessite formation) were used to evaluate Mn (II)-oxidation in the 109 endospore-forming strains. Only 18.35% of strains were positive in the screening on K medium at 10 µM of Mn (II). In contrast, more than 44% of the isolates exhibited Mn (II)-oxidizing activity through visible Birnessite formation in *Leptothrix* medium at 1 mM Mn (II).

In order to determine if MCOs are involved in Mn (II) oxidation in our collection of aerobic endospore-forming Firmicutes, the strains were screened for amplification of the copper-binding regions of *mnxG*, which are expected to be highly conserved due to their functional role. A ~900 bp product of *mnxG* was successfully amplified and verified by sequencing from 27.52% of the strains. Notably, only 11% of strains (12 strains) were positive for all three types of screening tests (Supplementary Table S2),

those included mainly strains belonging to *Bacillus* (8 strains), although strains belonging to *Paenibacillus* (1 strain), *Geobacillus* (2 strains) and *Exiguobacterium* (1 strain) were also detected. The same percentage of strains was positive for the amplification of *mnxG* but did not oxidize Mn (II) under the conditions of the culture assays. Among this second group, three *Anoxybacillus*, a *Geobacillus*, an *Aneurinibacillus* and an *Exiguobacterium* were detected besides *Bacillus*.

3.3. Cu (II) resistance/tolerance

The isolated endospore-forming Bacilli were then tested for copper resistance with different concentrations of Cu (II). From the total collection, 27 strains were not able to grow in presence of copper, seven of them could tolerate up to 0.5 mM of Cu (II), 25 up to 0.75 mM, 36 up to 1 mM, eight up to 1.5 mM, two up to 2 mM, one up to 2.5 mM, and three up to 3mM. Ten strains, however, were able to grow in presence of Cu (II) in the solid medium up to 2 mM of Cu (II) (Supplementary Table S3 & S4) and four highly resistant strains tolerated up to 3 mM of copper. Minimum inhibitory concentration (MIC) of Cu (II) on these resistant isolates was estimated in liquid medium amended with Cu (II) concentrations from 0.1 to 8 mM. MIC tests were performed in liquid since on solid media there is a potential diffusion bias. Two of these strains (Et 9/2 and Lr 5/4) showed the highest MIC (5 mM) for Cu (II).

3.4. 16S rRNA phylogeny and co-existence of Cu (II) resistance and Mn (II)-oxidation

The phylogenetic analysis based on the 16S rRNA gene of the 109 isolates was related to the physicochemical parameters measured in situ and to the biochemical tests on manganese oxidation and copper tolerance, as well as to the presence of a manganese oxidation biomarker (*mnxG*). This analysis revealed some interesting observations about the ecology of aerobic endospore-forming Firmicutes. First, the phylogeny of these strains is not related to their geographical distribution. The same species can be found in sampling sites that are very distant. Their distribution, however, is sometimes related to environmental conditions and to the similarity between habitats. For example, *Anoxybacillus rupiensis* and *Bacillus smithii* are found to colonize a specific ecological niche, that of geothermal reservoirs. *Exiguobacterium mexicanum* isolates all originated from a sulfur geothermal spring. Other species can be found in different geographical locations but also in different environmental conditions, for example *Bacillus cereus* was found in 11 habitats. A second observation made is that geographic location and manganese oxidation or copper resistance are not necessarily correlated, unless similar physicochemical parameters co-occur, for example isolates from Lirima, Chile, may or may not oxidize manganese depending on similarities in the temperature and pH measured at the sampling site. Likewise, these isolates exhibit different copper resistance maxima.

Among *Anoxybacillus* genus manganese oxidation was not observed (with the exception of strain Lr10/3), however, some *Anoxybacillus* isolates had the *mnxG* gene. On the contrary, manganese oxidation was observed in all *Lysinibacillus* species. This observation was not the case for other genera, such as *Geobacillus* and *Bacillus*, for which manganese oxidation, as well as presence of *mnxG* gene, depend on pH of the sampling site and not on phylogeny.

Among *Exiguobacterium,* copper tolerance seems to be species specific, as *Exiguobacterium aurantiacum* did not tolerate copper, while all strains that belong to *E. mexicanum* tolerated up to 1.5 mM of copper. Another species-secific observation is that isolates that belong to *Bacillus niacini*

showed a high copper tolerance. Finally, *Bacillus amylolyquefaciens* strains tolerated up to 1.5 mM of copper and oxidized manganese, although *mnxG* could not be amplified.

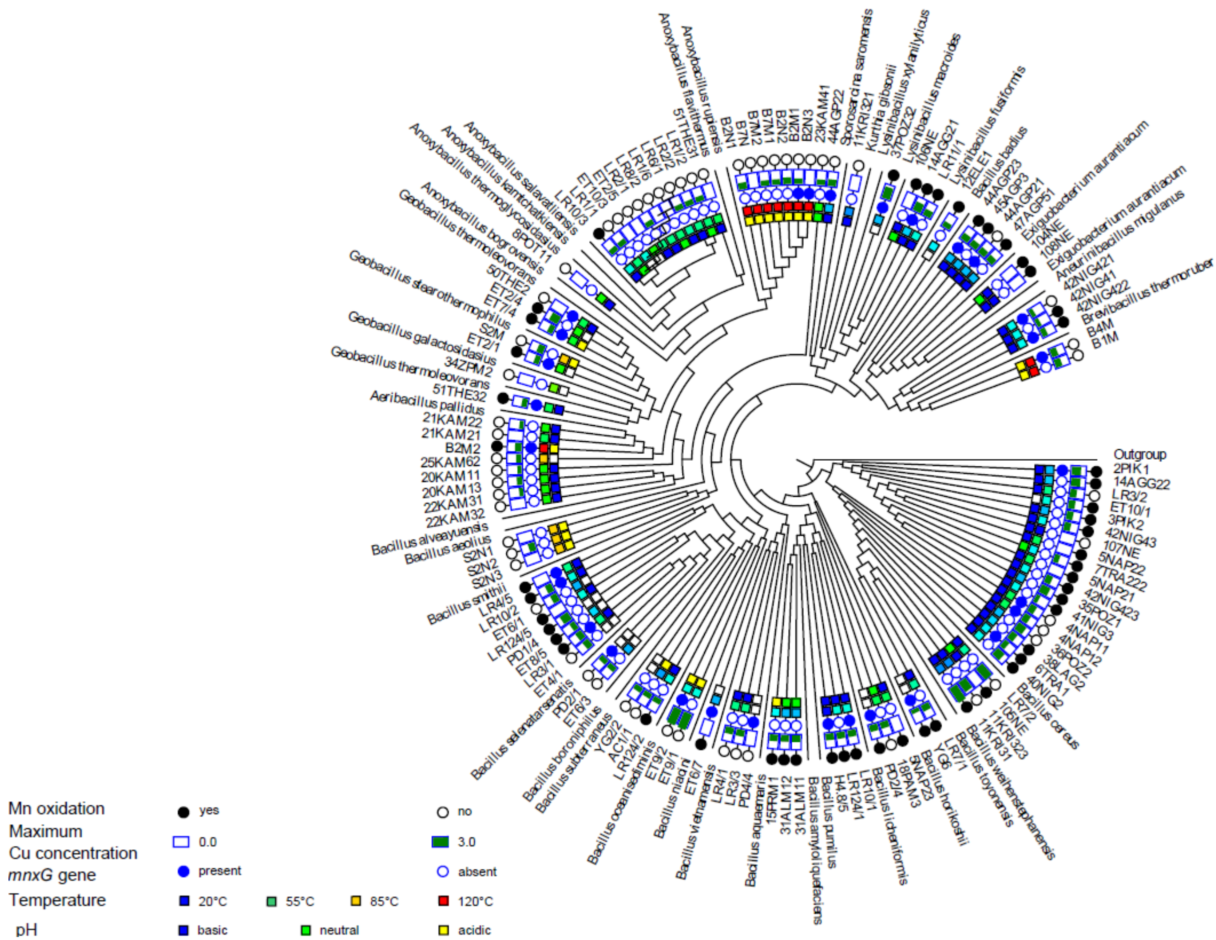

Figure 1. Maximum likelihood phylogenetic tree describing the phylogenetic relationship between isolates (code) and reference strains (species name indicated). On each branch of the tree, four parameters are indicated. Shape and color code for each parameter is described in the figure legend.

4. Discussion

In this study Mn (II) oxidation and copper tolerance were evaluated for a collection of aerobic endospore-forming Firmicutes isolated from a diverse set of natural uncontaminated sites. Despite the fact that research in metal tolerance is normally conducted in contaminated environments, uncontaminated sites might harbor a large diversity of microorganisms displaying different mechanisms of metal homeostasis.

As far as manganese oxidation is concerned, based on the results for Birnessite production, it can be inferred that uncontaminated environmental sites harbor an abundant population of Mn (II)-oxidizing aerobic endospore-forming Firmicutes. Mn (II)-oxidation is a metabolic trait found in a diverse set of unrelated microorganisms including representatives of the Alphaproteobacteria, Betaproteobacteria,

Gammaproteobacteria, as well as high GC Gram-positive Actinobacteria and low GC Gram-positive Firmicutes [37]. Mn (II)-oxidizers have been assigned to various phylogenetic lineages within *Bacillus* demonstrating a large diversity of species bearing this trait even within this confined genus [11,22]. This was also the case for our large collection of unrelated *Bacillus* strains. Moreover, our results show that Mn (II)-oxidation is a trait widely spread in other aerobic endospore-forming Firmicutes genera, since we have observed Mn (II)-oxidation in strains affiliated to other genera such as *Aeribacillus, Geobacillus, Anoxybacillus, Lysinibacillus, Exiguobacterium* and *Brevibacillus*.

Biogenic Mn oxides have recently gained in importance in environmental research. As nanoparticulate cryptocrystalline materials, biogenic Mn oxides are the most highly reactive Mn phases in the environment [10]. They participate in redox and sorption reactions that control the distribution of numerous other trace and contaminant elements (e.g. Ni [31], Pb [38]), as well as the sorption of organic matter [39]. Therefore, understanding the mechanisms of microbial mediated Mn (II)-oxidation process studied here could be significant in both biogeochemical and biotechnological contexts [40,41]. For many of the strains, amplification of a common genetic marker of multicopper oxidases (MCOs), which are so far identified as a key element of Mn (II)-oxidation [11,12] did not yield results, and thus further studies into the existence of alternative MCOs or other mechanisms of oxidation for the studied strains are still required.

In the case of resistance/tolerance of microorganisms to copper, overall, resistance decreased with increasing Cu (II) concentrations. Our results are consistent with a previous study that indicates that the number of resistant bacteria decreased at higher Cu (II) concentration [15].

Manganese oxidation and copper tolerance are specific in some genera, such as *Anoxybacillus* and *Lysinibacillus*, but this is not the case for all aerobic endospore-forming Firmicutes tested herein. This observation is also true for some species; however, others, like *Bacillus cereus,* do not exhibit a specific pattern concerning either manganese oxidation and copper resistance or geographical distribution and niche specialization. It is worth mentioning that a strain related to *Kurthia gibsonii* strain (11Kri-321) was positive for the screening with the two culture media but did not produce a PCR product for the *mnxG* gene with the primers evaluated here.

Our findings show that manganese oxidation in nature is not specific to bacterial species or site location. This is equally true for copper tolerance. A positive correlation between pH measured in situ and manganese oxidation was observed ($R^2 = 0.351$, p-value = $9.13e^{-05}$) as well as a negative correlation between temperature and Cu concentration (R^2 = 0.218, p-value = 0.027). These findings do not contradict the previously well-demonstrated relationship between biosorption and physicochemical parameters [42-44]. The results also suggests that endospore-formers have natural resistance mechanisms for toxic metals and that metal resistance is a wide spread phenomenon in endospore-forming Firmicutes. The potential for bioaccumalation/biosorption of toxic metals already suggested in the literature [43], opens up the possibility for use of these spore-formers in biological treatment processes, applied to effluents or sites contaminated with a wide range of toxic metals.

5. Conclusion

Aerobic endospore-forming Firmicutes isolated from natural, uncontaminated environments were found to oxidize manganese and resist up to 3 mM of copper. These capabilities were related to environmental physicochemical parameters, however, some genera and some species showed concrete

patterns of manganese oxidation and copper resistance. Not all strains able to oxidize manganese were copper tolerant, and vice-versa, and thus the existence of one capability cannot be assumed to be a prerequisite for the presence of the other. However, our initial hypothesis that manganese oxidation and copper tolerance are widespread phenomena among aerobic endospore-forming bacilli is confirmed.

Acknowledgments

We acknowledge funding from the Swiss National Funding projects 31003A_132358/1 and 31003A_152972, from Fondation Pierre Mercier pour la science and from REGARD for equality of women in science. We would like to thank the Swiss Confederation Fellowship Program for Foreign Researchers for funding S. Ganesan and S. Filippidou.

The genetic information downloaded from Genbank may be considered to be part of the genetic patrimony of Switzerland, Germany, France, Greece, Colombia and Chile respectively, the countries from which the samples were obtained. Users of this information agree to: 1) acknowledge Switzerland, Germany, France, Greece, Colombia and Chile as the countries of origin in any country where the genetic information is presented and 2) contact the CBD focal point and the ABS focal point identified in the CBD website http://www.cbd.int/information/nfp.shtml if they intend to use the genetic information for commercial purposes.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary

Table S1. Sampling sites and their description.

Sampling site	Country	Site description	Temperature	pH
Neuchatel	Switzerland (CH)	Mineral iron and sulfur springs	15	6.94–7.13
Soultz-sous-forets	France (FR)	Geothermal power plant	100	5.2
Bruschal	Germany (DE)	Geothermal power plant	122	5.4
Salar de Yungai	Chile (CL)	Salt crusts	n/a	n/a
Aguas Calientes	Chile (CL)	Geothermal natural spring	32	6
Laguna de las Piedras	Chile (CL)	Atakama Lake	n/a	n/a
El Tatio	Chile (CL)	Geysers	34–70	5.5
Salar de Huasco	Chile (CL)	Lagoon of thermal or cold inlets	15.8	8.6
Lirima	Chile (CL)	Thermal site with springs and streams	45-55	7.48–8.04
Lagadas	Greece (GR)	Geothermal natural spring	38	8
Pikrolimni	Greece (GR)	Lake	35	9.21–9.86
Nea Appolonia	Greece (GR)	Geothermal natural spring	59	8.2–8.8
Milos	Greece (GR)	Volcanic island	35–100	6.13–7.5
Agia Paraskevi	Greece (GR)	Mineral sulfur spring	35	7.6
Thermia	Greece (GR)	Geothermal natural spring	60	7.6
Nigrita	Greece (GR)	Geothermal natural spring	43	8.2
Pozar	Greece (GR)	Geothermal natural spring	35	7.7–8.3
Aggistro	Greece (GR)	Geothermal natural spring	37	8.1
Eleftheres	Greece (GR)	Geothermal natural spring	41	n/a
Krinides	Greece (GR)	Mineral spring	30	7.99
Potamia	Greece (GR)	Geothermal natural spring	70	8.55
Traianoupoli	Greece (GR)	Geothermal natural spring	41	7.34–7.56

Table S2. Strains isolated per sampling site (Origin) and identified against EzTaxon's cultured database (Closest relative). Manganese oxidation capability on two different media, presence of mnGx gene and copper tolerance at different concentrations is also presented. Co-presence of manganese oxidation capability and copper tolerance is shown in column Mn-Cu.

Nr	Strains	Origin	Closest relative	Manganese (II) Oxidation			Copper (II) Tolerance							Mn-Cu
				K medium	Birnessite	MnOx gene	Copper supplemented medium (Cu II concentration in mM)							
							0.5 mM	0.75 mM	1.0 mM	1.5 mM	2.0 mM	2.5 mM	3.0 mM	
1	107NE	Neuchatel	*Bacillus cereus*	-	-	-	-	-	-	-	-	-	-	-
2	21KAM21	Kalamos-Milos	*Aeribacillus pallidus*	-	-	-	-	-	-	-	-	-	-	-
3	22KAM32	Kalamos-Milos	*Bacillus alveayuensis*	-	-	-	-	-	-	-	-	-	-	-
4	34ZPM2	Zefiria Milos	*Geobacillus galactosidasius*	-	-	-	-	-	-	-	-	-	-	-
5	51THE31	Thermia	*Anoxybacillus flavithermus*	-	-	-	-	-	-	-	-	-	-	-
6	8POT11	Potarmia	*Anoxybacillus flavithermus*	-	-	-	-	-	-	-	-	-	-	-
7	B2N3	Bruschal	*Anoxybacillus rupiensis*	-	-	-	-	-	-	-	-	-	-	-
8	B7N	Bruschal	*Anoxybacillus rupiensis*	-	-	-	-	-	-	-	-	-	-	-
9	Et10/3	El Tatio	*Anoxybacillus salavatliensis*	-	-	-	-	-	-	-	-	-	-	-
10	Et2/5	El Tatio	*Anoxybacillus bogrovensis*	-	-	-	-	-	-	-	-	-	-	-
11	Lr1/6	Lirima	*Anoxybacillus salavatliensis*	-	-	-	-	-	-	-	-	-	-	-
12	Lr4/5	Lirima	*Bacillus boroniphilus*	-	-	-	-	-	-	-	-	-	-	-
13	Lr8/2	Lirima	*Anoxybacillus salavatliensis*	-	-	-	-	-	-	-	-	-	-	-
14	S2N1	Soultz	*Bacillus smithi*	-	-	-	-	-	-	-	-	-	-	-
15	S2N3	Soultz	*Bacillus smithi*	-	-	-	-	-	-	-	-	-	-	-
16	104NE	Neuchatel	*Exiguobacterium aurantiacum*	+	-	-	-	-	-	-	-	-	-	-
17	105NE	Neuchatel	*Bacillus thuringiensis*	-	+	-	-	-	-	-	-	-	-	-
18	106NE	Neuchatel	*Lysinibacillus macroides*	-	+	-	-	-	-	-	-	-	-	-
19	20KAM11	Kalamos-Milos	*Bacillus alveayuensis*	-	-	-	+	-	-	-	-	-	-	-
20	21KAM22	Kalamos-Milos	*Aeribacillus pallidus*	-	-	-	+	-	-	-	-	-	-	-

21	22KAM31	Kalamos-Milos	*Bacillus alveayuensis*	-	-	-	+	-	-	-	-	-	-	-
22	5NAP21	Nea Apollonia	*Bacillus cereus*	-	+	-	-	-	-	-	-	-	-	-
23	5NAP22	Nea Apollonia	*Bacillus cereus*	-	+	-	-	-	-	-	-	-	-	-
24	5NAP23	Nea Apollonia	*Bacillus licheniformis*	-	-	+	-	-	-	-	-	-	-	-
25	Lr124/5	Lirima	*Bacillus jeotgali*	+	-	-	-	-	-	-	-	-	-	-
26	Pd4/4	Las Piedras	*Bacillus aquimaris*	-	-	+	-	-	-	-	-	-	-	-
27	108NE	Neuchatel	*Exiguobacterium mexicanum*	+	+	-	-	-	-	-	-	-	-	-
28	11KRI321	Krinides	*Kurthia gibsonii*	+	+	-	-	-	-	-	-	-	-	-
29	20KAM13	Kalamos-Milos	*Bacillus alveayuensis*	-	-	-	+	+	-	-	-	-	-	-
30	23KAM41	Kalamos-Milos	*Bacillus aeolius*	-	-	-	+	+	-	-	-	-	-	-
31	25KAM62	Kalamos-Milos	*Aeribacillus pallidus*	-	-	-	+	+	-	-	-	-	-	-
32	41NIG3	Nigrita	*Bacillus cereus*	-	-	+	+	-	-	-	-	-	-	-
33	42NIG41	Nigrita	*Aneurinibacillus migulanus*	-	+	-	+	-	-	-	-	-	-	+
34	42NIG421	Nigrita	*Aneurinibacillus migulanus*	-	+	-	+	-	-	-	-	-	-	+
35	50THE2	Thermia	*Geobacillus thermoleovorans*	-	+	+	-	-	-	-	-	-	-	-
36	Ac1/1	Aguas Calientes	*Bacillus oceanisediminis*	-	-	-	+	+	-	-	-	-	-	-
37	B7M1	Bruschal	*Anoxybacillus rupiensis*	-	-	-	+	+	-	-	-	-	-	-
38	B7M2	Bruschal	*Anoxybacillus rupiensis*	-	-	-	+	+	-	-	-	-	-	-
39	Et6/3	El Tatio	*Bacillus boroniphilus*	-	-	-	+	+	-	-	-	-	-	-
40	Lr1/1	Lirima	*Anoxybacillus salavatliensis*	-	-	-	+	+	-	-	-	-	-	-
41	Lr1/2	Lirima	*Anoxybacillus thermarum*	-	-	-	+	+	-	-	-	-	-	-
42	Lr2/1	Lirima	*Anoxybacillus kamchatkensis*	-	-	-	+	+	-	-	-	-	-	-
43	Lr2/2	Lirima	*Anoxybacillus thermarum*	-	-	-	+	+	-	-	-	-	-	-
44	Lr6/1	Lirima	*Anoxybacillus bogrovensis*	-	-	-	+	+	-	-	-	-	-	-
45	Yg2/2	Yungai	*Bacillus oceanisediminis*	-	-	-	+	+	-	-	-	-	-	-
46	12ELE1	Eleftheres	*Bacillus badius*	-	+	-	+	+	-	-	-	-	-	+
47	18PAM3	Palaiochori Milos	*Bacillus sp.*	-	-	-	+	+	+	-	-	-	-	-
48	51THE32	Thermia	*Geobacillus thermoglucosidasius*	-	-	+	+	+	-	-	-	-	-	-

49	7TRA222	Traianoupoli	*Bacillus cereus*	-	+	-	+	+	-	-	-	-	-	+
50	B1M	Bruschal	*Brevibacillus thermoruber*	-	-	-	+	+	+	-	-	-	-	-
51	B2M1	Bruschal	*Anoxybacillus rupiensis*	-	-	+	+	+	-	-	-	-	-	-
52	B4M	Bruschal	*Brevibacillus thermoruber*	-	-	-	+	+	+	-	-	-	-	-
53	Et4/1	El Tatio	*Bacillus thioparans*	-	-	-	+	+	+	-	-	-	-	-
54	Et6/1	El Tatio	*Bacillus thioparans*	-	-	-	+	+	+	-	-	-	-	-
55	Et6/7	El Tatio	*Bacillus vietnamensis*	+	+	+	-	-	-	-	-	-	-	-
56	Et8/5	El Tatio	*Bacillus jeotgali*	+	-	-	+	+	-	-	-	-	-	+
57	Lr124/2	Lirima	*Bacillus oceanisediminis*	-	+	-	+	+	-	-	-	-	-	-
58	Lr3/1	Lirima	*Bacillus thioparans*	-	-	-	+	+	+	-	-	-	-	-
59	Lr3/2	Lirima	*Bacillus cereus*	-	-	-	+	+	+	-	-	-	-	-
60	Pd2/1	Las Piedras	*Bacillus boroniphilus*	-	-	+	+	+	-	-	-	-	-	-
61	S2M	Soultz	*Geobacillus stearothermophilus*	-	-	-	+	+	+	-	-	-	-	-
62	S2N2	Soultz	*Bacillus smithi*	-	-	-	+	+	+	-	-	-	-	-
63	15PRM1	Provtas-Milos	*Bacillus subtilis*	-	+	-	+	+	+	-	-	-	-	+
64	31ALM11	Milos Liogerma	*Bacillus amylolyquefaciens*	-	+	-	+	+	+	-	-	-	-	+
65	31ALM12	Milos Liogerma	*Bacillus amylolyquefaciens*	-	+	-	+	+	+	-	-	-	-	+
66	38LAG2	Lagadas	*Bacillus cereus*	-	+	-	+	+	+	-	-	-	-	+
67	3PIK2	Pikrolimni	*Bacillus cereus*	-	+	-	+	+	+	-	-	-	-	+
68	42nig43	Nigrita	*Bacillus cereus*	-	+	-	+	+	+	-	-	-	-	+
69	44AGP21	Agia Paraskevi	*Exiguobacterium mexicanum*	-	-	+	+	+	+	-	-	-	-	-
70	44AGP22	Agia Paraskevi	*Sporosarcina saromensis*	-	+	-	+	+	+	-	-	-	-	+
71	47AGP51	Agia Paraskevi	*Exiguobacterium aurantiacum*	-	-	+	+	+	+	-	-	-	-	-
72	4NAP11	Nea Apollonia	*Bacillus cereus*	-	+	-	+	+	+	-	-	-	-	+
73	4NAP12	Nea Apollonia	*Bacillus thuringiensis*	-	-	+	+	+	+	-	-	-	-	-
74	B2M2	Bruschal	*Aeribacillus pallidus*	-	-	+	+	+	+	-	-	-	-	-
75	B2N1	Bruschal	*Anoxybacillus rupiensis*	-	-	+	+	+	+	-	-	-	-	-
76	Et10/1	El Tatio	*Bacillus thuringiensis*	-	+	-	+	+	+	-	-	-	-	+

77	Lr10/1	Lirima	*Bacillus licheniformis*	-	+	-	+	+	+	-	-	-	-	+
78	Lr10/3	Lirima	*Anoxybacillus thermarum*	+	-	+	+	+	-	-	-	-	-	+
79	Lr11/1	Lirima	*Lysinibacillus fusiformis*	-	+	-	+	+	+	-	-	-	-	+
80	Lr124/1	Lirima	*Bacillus licheniformis*	-	+	-	+	+	+	-	-	-	-	+
81	Lr7/1	Lirima	*Bacillus cereus*	-	+	-	+	+	+	-	-	-	-	+
82	Yg6	Yungai	*Bacillus horikoshii*	+	+	+	+	-	-	-	-	-	-	+
83	11KRI323	Krinides	*Bacillus toyonensis*	-	-	-	+	+	+	+	+	-	-	-
84	14AGG22	Aggistro	*Bacillus cereus*	-	+	-	+	+	+	+	-	-	-	+
85	35POZ1	Pozar	*Bacillus cereus*	-	+	-	+	+	+	+	-	-	-	+
86	37POZ32	Pozar	*Lysinibacillus xylanilyticus*	-	-	-	+	+	+	+	+	-	-	-
87	40NIG2	Nigrita	*Bacillus thuringiensis*	-	+	-	+	+	+	+	-	-	-	+
88	42NIG422	Nigrita	*Bacillus sp.*	+	+	+	+	+	-	-	-	-	-	+
89	42NIG423	Nigrita	*Bacillus cereus*	+	+	-	+	+	+	-	-	-	-	+
90	44AGP23	Agia Paraskevi	*Exiguobacterium sp.*	+	+	-	+	+	+	-	-	-	-	+
91	6TRA1	Traianoupoli	*Bacillus sp.*	+	+	+	+	+	-	-	-	-	-	+
92	B2N2	Bruschal	*Anoxybacillus rupiensis*	-	+	+	+	+	+	-	-	-	-	+
93	Et2/1	El Tatio	*Geobacillus sp.*	+	+	+	+	+	-	-	-	-	-	+
94	Et2/4	El Tatio	*Geobacillus thermoparaffinivorans*	-	+	-	+	+	+	+	-	-	-	+
95	Et7/4	El Tatio	*Geobacillus stearothermophilus*	+	+	+	+	+	-	-	-	-	-	+
96	Lr10/2	Lirima	*Bacillus subterraneus*	-	+	+	+	+	+	-	-	-	-	+
97	Pd1/4	Las Piedras	*Bacillus selenatarsenatis*	-	+	-	+	+	+	+	-	-	-	+
98	14AGG21	Aggistro	*Lysinibacillus sp.*	-	+	+	+	+	+	+	-	-	-	+
99	2PIK1	Pikrolimni	*Bacillus cereus*	-	+	+	+	+	+	+	-	-	-	+
100	45AGP3	Agia Paraskevi	*Exiguobacterium sp.*	+	+	+	+	+	+	-	-	-	-	+
101	H4.8/5	Huasco	*Bacillus pumilus*	+	+	+	+	+	+	-	-	-	-	+
102	Lr3/3	Lirima	*Bacillus aquimaris*	+	+	+	+	+	+	-	-	-	-	+
103	Lr4/1	Lirima	*Bacillus aquimaris*	+	+	+	+	+	+	-	-	-	-	+
104	Lr7/2	Lirima	*Bacillus thuringiensis*	-	-	-	+	+	+	+	+	+	-	-

105	Pd2/4	Las Piedras	*Bacillus licheniformis*	+	+	+	+	+	+	-	-	-	-	+
106	11KRI31	Krinides	*Bacillus weihenstephanensis*	-	-	-	+	+	+	+	+	+	+	-
107	36POZ2	Pozar	*Bacillus cereus*	+	+	+	+	+	+	+	-	-	-	+
108	Et9/1	El Tatio	*Bacillus niacini*	-	-	-	+	+	+	+	+	+	+	-
109	Et9/2	El Tatio	*Bacillus niacini*	-	-	+	+	+	+	+	+	+	+	+

Table S3. (II) biosorption and pH change during the growth of high Cu (II) tolerant endospore-forming Firmicutes. MIC= Minimum inhibitory concentration.

			Cu II tolerant strains & their pH change, final Cu II; Control pH 7.2 ± 0.2 (0.0 mM Cu II)																			
Cu II mM	Initial Cu II (mg/L)	Initial pH	Et 9/1		Et 9/2		Lr 5/4		Lr 7/2		9kri1		10kri2		11kri31		11kri323		11kri324		37poz32	
			Final Cu II	Final pH	Final Cu II	Final pH	Final Cu II	Final pH	Final Cu II	Final pH	Final Cu II	Final pH	Final Cu II	Final pH	Final Cu II	Final pH	Final Cu II	Final pH	Final Cu II	Final pH	Final Cu II	Final pH
0.1	24.97	7.08	0.099	7.35	0.072	7.44	0.066	8.25	0.099	7.14	0.070	7.11	0.380	7.44	0.068	4.91	0.077	7.66	0.086	8.38	0.118	8.57
0.25	62.425	7.02	0.260	7.10	0.312	7.57	0.355	8.29	0.197	7.83	0.136	7.46	0.251	7.78	0.258	4.97	0.154	5.09	0.263	8.04	0.367	8.42
0.5	124.85	6.90	0.298	8.22	0.506	8.22	0.543	8.51	0.479	7.54	0.556	7.14	0.603	7.65	0.561	5.05	0.340	7.43	0.492	7.96	0.602	8.58
0.75	187.275	6.80	0.448	6.68	0.746	7.42	0.747	8.21	0.679	7.87	0.828	7.02	0.985	7.55	0.749	5.10	0.582	7.94	0.611	7.28	0.875	8.46
1.0	249.7	6.70	0.979	6.79	0.692	7.19	1.011	8.27	0.889	7.26	1.082	7.28	1.112	7.30	0.969	5.07	0.703	8.28	0.749	7.34	0.686	7.98
1.5	374.55	6.45	0.675	7.91	0.993	6.31	0.735	7.86	0.765	7.12	1.167	6.98	1.048	7.54	1.055	5.18	0.851	5.24	0.647	7.31	0.461	8.55
2.0	499.4	6.22	0.975	6.25	0.757	7.25	0.579	7.49	0.842	5.68	1.144	6.98	0.652	5.67	0.757	5.01	0.731	5.40	0.727	7.49	0.588	5.86
2.5	624.25	6.02	0.816	6.87	0.623	7.42	0.323	7.88	0.948	5.70	1.155	6.74	0.929	5.29	0.649	5.06	0.689	5.40	0.824	7.13	0.829	6.90
3.0	749.1	5.78	0.854	5.22	0.796	6.0	0.857	5.06	0.778	5.47	1.152	5.65	0.639	4.77	0.673	4.97	0.463	6.10	0.616	6.23	MIC	
3.5	873.95	5.55	0.746	5.47	0.774	4.75	0.717	4.73	MIC		1.157	5.59	MIC		MIC		MIC		MIC			
4.0	998.8	5.35	MIC		0.811	5.09	0.726	4.75			MIC											
5.0	1248.5	4.97			MIC		MIC															

Table S4. Growth in high Cu (II) tolerant endospore-forming bacteria. Growth observed (+).

Strains	Growth with metal (Cu II mM)		MIC-Cu II (mM)
	Solid media	Liquid media	
Et 9/1	3.0++	3.5+	4.0
Et 9/2	3.0+	4.0+	5.0
Lr 5/4	2.0+	4.0+	5.0
Lr 7/2	2.5+	3.0++	3.5
9kri 1	2.5+	3.5+	4.0
10kri 2	2.0+	3.0++	3.5
11kri 31	3.0+	3.0++	3.5
11kri323	2.0+	3.0++	3.5
11kri324	3.0+	3.0++	3.5
37poz32	2.0+	2.5+	3.0

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