Complete genome sequencing and comparative genomic analyses of Bacillus sp. S3, a novel hyper Sb(III)-oxidizing bacterium

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Abstract

Background: Antimonite [Sb(III)]-oxidizing bacterium has great potential in the environmental bioremediation of Sb-polluted sites. Bacillus sp. S3 that was previously isolated from antimony-contaminated soil displayed high Sb(III) resistance and Sb(III) oxidation efficiency. However, the genomic information and evolutionary feature of Bacillus sp. S3 are very scarce.

Results: Here, we identified a 5,436,472 bp chromosome with 40.30% GC content and a 241,339 bp plasmid with 36.74% GC content in the complete genome of Bacillus sp. S3. Genomic annotation showed that Bacillus sp. S3 contained a key aioB gene potentially encoding As (III)/Sb(III) oxidase, which was not shared with other Bacillus strains. Furthermore, a wide variety of genes associated with Sb(III) and other heavy metal (loid)s were also ascertained in Bacillus sp. S3, reflecting its adaptive advantage for growth in the harsh eco-environment. Based on the analysis of phylogenetic relationship and the average nucleotide identities (ANI), Bacillus sp. S3 was proved to be a novel species within the Bacillus genus. The majority of mobile genetic elements (MGEs) mainly distributed on chromosomes within the Bacillus genus. Pan-genome analysis showed that the 45 genomes contained 554 core genes and many unique genes were dissected in analyzed genomes. Whole genomic alignment showed that Bacillus genus underwent frequently large-scale evolutionary events. In addition, the origin and evolution analysis of Sb(III)-resistance genes revealed the evolutionary relationships and horizontal gene transfer (HGT) events among the Bacillus genus. The assessment of functionality of heavy metal (loid)s resistance genes emphasized its indispensable role in the harsh eco-environment of Bacillus genus. Real-time quantitative PCR (RT-qPCR) analysis indicated that Sb(III)-related genes were all induced under the Sb(III) stress, while arsC gene was down-regulated.

Conclusions: The results in this study shed light on the molecular mechanisms of Bacillus sp. S3 coping with Sb(III), extended our understanding on the evolutionary relationships between Bacillus sp. S3 and other closely related species, and further enriched the Sb(III) resistance genetic data sources.

Keywords: Bacillus sp. S3, Sb(III)-resistance, Genome sequencing, Comparative genome, Heavy metal (loid)s

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Background
The hazardous heavy metal (loid) s, such as antimony (Sb), arsenic (As), cadmium (Cd), chromium (Cr) and lead (Pb) exert a serious threat to the natural environments and human health in many parts of the world [1–3]. In recent decades, natural biogeochemical cycle and anthropogenic activities including mining activities, rapid urbanization, and industrialization have contributed to elevated levels of heavy metal (loid) s in soils [4, 5]. Conventional remediation technologies have been developed to remove heavy metal (loid) s from contaminated surroundings, such as ion exchange, membrane separation, coagulation/flocculation, electrochemical methods, extraction and adsorption [6, 7]. However, most of these physiochemical remediation methods are not suitable for large-scale applications because of their high cost, generation of secondary pollution and unsustainable nature [8]. By contrast, microorganism-mediated bioremediation is an alternative promising technology due to its low-cost and environmentally friendly advantages [9]. Microorganisms are able to alleviate the toxicity of heavy metal (loid) s using various resistance strategies, such as extracellular precipitation, intracellular sequestration, enzymatic transformation and oxido-reduction of toxic metal ions [8, 10].

Antimony (Sb) is a toxic metalloids in group 15 of the periodic table of elements and excessive Sb can cause harmful damages to the human health [1, 4]. Sb and its compounds have been listed as priority pollutants by the United States Environmental Protection Agency (USEPA) and the European Union (EU) [7, 11]. The maximum acceptable concentration of Sb in drinking water has been set at 6 μg/L by the World Health Organization (WHO) [12]. Because it’s widely used in flame retardants, Pb-Sb alloys, brake linings, and catalysts for polyethylene glycol terephthalate [4, 13], a sharply increasing release of Sb in the environments occurs during the past decades [1]. The main Sb species include antimone [(Sb(III)] and antimonate [Sb(V)] in soil and water systems, which can be interconverted via biogeochemical processes. Sb(V) is more stable in aerobic environments than Sb(III), and Sb(III) is more toxic than Sb(V) due to its high affinity with thiol-containing proteins [14]. Thus, microbial Sb(III) oxidation that can transform the toxic Sb(III) to the less toxic Sb(V) has a significant value for the environmental Sb bioremediation [15].

In recently, more than 60 Sb(III)-oxidizing bacteria, including Shinella sp. strain NLS1, Ensifer sp. strain NLS4, Acinetobacter sp. JL7, Comamonas sp. JL25, Comamonas sp. JL40, Comamonas sp. S44, Stenotrophomonas sp. JL9, and Bosea sp. AS-1 have been isolated from different Sb-contaminated sites [7, 13, 14, 16]. Bacillus sp. S3 is a new hyper antimony-oxidizing bacterium, which has been previously isolated from contaminated mine soils [17]. Our previous study confirmed that it exhibited high Sb(III) oxidation efficiency (50 μM·d−1) and Sb(III) resistance (5.5 mM) [17]. Meanwhile, this bacterial strain has been proved to occupy the ability to cope with multiple heavy metal (loid) s through various adaptive strategies [18]. Although increasing numbers of studies have focused on microbial Sb oxidation, the mechanisms of Sb transformation in Bacillus sp. S3 have not been well explored so far. Meanwhile, the lack of data on its genome sequence has restricted molecular studies and practical applications.

Furthermore, it is generally noted that Bacillus is a large genus of the gram-positive, heterotrophic, endospore-forming bacteria and belongs to Class: Bacilli, Phylum: Firmicutes [19]. Members of the Bacillus genus provide a model system for the study of metal ions and exhibit broad resistance to heavy metals [20]. With the extending of the third-generation sequencing platform PacBio RSII, large numbers of the Bacillus strains (more than 4551) have been genomic sequenced. In contrast, there are few literatures on Sb(III) oxidation and resistance mechanisms in Bacillus genus. The arsenite oxidase AioBA responsible for As (III) oxidation in Agrobacterium tumefaciens 5A was reported to also function as a Sb(III) oxidase [12]. Moreover, arsenite oxidase AioAB is composed of a large (AioA) and a small (AioB) subunit [21]. A novel Sb(III) oxidase AnoA was discovered to catalyze Sb(III) oxidation in Agrobacterium tumefaciens GW4 with NADP+ as the co-factor [22], the cellular H2O2 catalyzed bacterial Sb(III) oxidation as an abiotic oxidant [23]. Although these studies provide great advance, there has never been a comprehensive research focused on Sb(III) oxidation in terms of whole genome and comparative genomics. To further understand the molecular details of Bacillus sp. S3 against Sb(III), it is essential to determine the genomic information of Sb(III)-resistance strains. Here, genomes sequence and the comparative genomic analyses were applied to study the Sb resistance mechanism and evolutionary relationship of Bacillus sp. S3.

Results
Morphological characterization of Bacillus sp. S3
As shown in Fig. 1a, the SEM images of Bacillus sp. S3 intuitively showed that the cell walls were enveloped by filaments, possibly due to the presence of extracellular polymeric substances. Intriguingly, after cultivation of Bacillus sp. S3 for exponential phase under the Sb(III) stress, the cell surface became smoother than control (Fig. 1b). Meanwhile, smaller cell size, lesser wrinkled cell wall and the occurrence of intracellular dissolution were visible in present of other heavy metal (loid) s (Fig. 1c-h). As shown in Figure S1A, no physical Sb(III) adsorption was detected on Bacillus sp. S3 cell surfaces by
EDS analysis, which was similar to other studies [9]. When the initial concentration was 1 mM Pb (II), the peak value of EDS was significant from that of the control group and other treatment groups (Figure S1E).

**General genome feature of Bacillus sp. S3**

The general genomic features of *Bacillus* sp. S3 were summarized in Table 1 and Fig. 2. The whole genome of *Bacillus* sp. S3 contained one single circular chromosome of 5,436,472 bp with 40.30% GC content and one plasmid of 241,339 bp with 36.74% GC content (Fig. 2a, b). The whole genome harbored 5131 protein-coding sequences covering 85.35% of the genome with the average length of 904 bp, as well as 36 rRNAs and 104 tRNAs (Table 1). In addition, the genomic features of *Bacillus* sp. S3 and the other 44 closely related strains were
showed lower GC contents (35.4%). B. thuringiensis tent (47.8%), and Bacillus strains, lar genomic characteristics. Among these investigated substantial inter-species or inter-strain variations and similar genomic features. The average chromosome length of the 45 Bacillus genomes was 4.99 Mb with ranging from 2.2 to 7.08 Mb and the average GC content was 40.30% with ranging from 35.4 to 47.8%, indicating substantial inter-species or inter-strain variations and similar genomic characteristics. Among these investigated strains, Bacillus sp. OxB-1 showed the highest GC content (47.8%), and B. thuringiensis and B. cereus S1 showed lower GC contents (35.4%).

Based on BLASTP searches (e value <1e^-5), there were 3833 and 2563 CDSs involved in COG database (Figure S2; Table S1) and GO database (Figure S3; Table S2), respectively. A high proportion of genes in COG database were assigned to the general function prediction only (R, 10.3%), amino acid transport and metabolism (E, 8.05%), carbohydrate transport and metabolism (G, 6.15%), energy production and conversion (C, 5.33%), transcription (K, 5.06%), and replication, recombination, and repair (L, 4.6%) categories. In addition, the proteins were distributed to GO database in three functional classification, including “molecular function” (2715), “cellular component” (1001) and “biological process” (3615). Compared to other bacteria, enrichment profiles of Bacillus sp. S3 genes assigned to COG functional categories showed an overabundance of genes involved in amino transport and metabolism, carbohydrate transport and metabolism, energy production and conversion. These resistance genes, such as ABC antiporters and Cd^{2+}/Zn^{2+}/Co^{2+} efflux components (CzcABC, CzcD), were probably important for Bacillus sp. S3 in the adaption of specific niche.

In addition, the KEGG pathway database was used to map their corresponding terms on the Bacillus sp. S3 genome. A total of 1195 CDSs were assigned to 180 KEGG pathways (Figure S4; Table S3), which could enrich in “Metabolism” (637), “Biosynthesis of secondary metabolites” (285), “Microbial metabolism in diverse environments” (215), “Carbon metabolism” (135), “Biosynthesis of amino acids” (134) and “ABC transporters” (122). The arsenite oxidase (AioB) was assigned to metabolic pathway (ko01100), the phosphate-binding protein (PstS), sn-glycerol-3-phosphate-binding periplasmic protein (UgpB) and phosphate import ATP-binding protein (PstB) were assigned to ABC transporters pathway (ko02010). The copper-ion-binding protein CopZ was assigned to mineral absorption pathways (ko04978). These pathways might play an important role in coping with the metal (loid) toxicity in Bacillus sp. S3.

As shown in Figure S5, we identified 237 carbohydrate-active enzymes (CAZymes) in the genome of Bacillus sp. S3. Predicted CAZymes were classified into 6 classes, encompassing auxiliary activities (AAs, 4), carbohydrate-binding modules (CBM, 63) carbohydrate esterases (CEs, 39), glycoside hydrolases (GHs, 90), glycosyltransferases (GTs, 39), polysaccharide lyases (PLs, 2). To induce inhibition, the heavy metal (loid) ions may non-specifically bind to regions of CAZymes [24]. This interactions between heavy metal (loid) s and CAZymes need a great deal of energy (e.g. for pumping out intracellular metal ions by ATPases, or for the strengthened expression of metal (loid) resistance proteins, etc.) through increased conversion of carbohydrates [25]. It implied that these enzymes might play an important role in coping with the metal (loid) ions [24].

Notably, large numbers of heavy metal (loid) resistance genes were found to locate on the chromosome rather than plasmid in Bacillus sp. S3 (Table 3), which could be used for further analysis of genetic diversity and evolution. Unlike chromosome, the plasmid was found to contain large numbers of hypothetical proteins. In our study, the aioB gene encoding Sb(III)/As (III) oxidase was only detected in the chromosome of Bacillus sp. S3 and not the other Bacillus genomes, giving Bacillus sp. S3 the capacity to oxidize Sb(III). Moreover, three arsB genes encoding Sb(III)/As (III) efflux pump membrane proteins and arsC gene encoding As(V) reductase were located on the chromosome in Bacillus sp. S3. As resistance genes (arsB and arsC) were detected in all comparative Bacillus strains, implying that cytoplasmic Sb(III) extrusion was the main As/Sb resistance strategy in Bacillus genus. The phosphate (Pi) related genes such as phoB, pstS, and phoK were also identified in Bacillus sp. S3, which have been proved to be co-regulated with As (III) oxidation and could be induced by Sb(III) in previous report [26].

**Phylogenetic analysis and ANI calculations**

To evaluate the phylogenetic relationship, we downloaded 44 Bacillus genome sequences (including 16
complete genomes) and their annotations from the NCBI database (Table 2). The phylogenetic tree based on 16S rRNA gene sequences revealed that Bacillus sp. S3 belonged to Bacillus genus and grouped with Bacillus sp. L75, Bacillus soli strain G8 and Bacillus drentensis G18 (Fig. 3a). The phylogenetic trees based on 554 core genes and whole-genome composition vectors (CVs) were also constructed (Fig. 3b, c). In the two phylogenetic trees, Bacillus sp. S3 grouped with Bacillus bataviensis, Bacillus soli, Bacillus novalis, Bacillus vireti, indicating that Bacillus sp. S3 was closest to the Bacillus bataviensis. However, the topologies of the two phylogenetic trees exhibited some differences, suggesting that the flexible genes could be crucial in altering the genome content and shaping the topology of the trees. As shown in Table 4, the closest ANI values 81.51% between Bacillus sp. S3 and other reference strains considerably lower than threshold value of 95–96% of the boundary for species circumscription [27]. Data illustrated in Table S4 showed that the dDDH% values of Bacillus sp. S3 against all reference genomes ranged from 12.7 to 34%. Therefore, the combination of ANI values and dDDH values showed that Bacillus sp. S3 belonged to novel species within the genera of Bacillus.

Fig. 2 Circular genome maps of Bacillus sp. S3 chromosome (a) and plasmid (b). From the outer to the inner circle: (1) scale marks of the genome; (2) assigned COG classes of protein-coding genes (CDSs) on the forward strand as indicated by relevant colors; (3) forward strand CDSs; (4) tRNA (black) and rRNA (red) genes on the forward strand; (5) tRNA (black) and rRNA (red) genes on the reverse strand; (6) GC content (swell outward/inward indicates higher/lower G + C compared with the average G + C content); (7) GC skew (cyan/red indicate positive/negative values).
| No. | Organism                  | NCBI accession       | Level         | Size (Mb) | GC%  | No. of Genes | No. of Proteins | rRNA | tRNA |
|-----|---------------------------|----------------------|---------------|-----------|------|--------------|----------------|------|------|
| 1   | *B. asahii* OM18           | NZ_CP026095.1        | Complete      | 4.89      | 37.45| 4,824        | 4,419          | 54   | 150  |
| 2   | *B. bataviensis* LMG 21833 | AILS00000000.1       | Contig        | 5.37      | 39.6 | 5,238        | 5,207          | –    | 23   |
| 3   | *B. cereus* S1             | ADFM00000000.1       | Contig        | 5.16      | 35.4 | 2,192        | 2,184          | –    | 8    |
| 4   | *B. cucumis* V32-6         | PGVE00000000.1       | Contig        | 5.71      | 38.6 | 5,552        | 5309           | 10   | 129  |
| 5   | *B. delmoensis* FF4(T)     | NZ_ADCA00000000.1    | Complete      | 4.57      | 40.9 | 4,433        | 4,184          | 20   | 136  |
| 6   | *B. drentensis* 102427     | BCUX00000000.1       | Contig        | 5.16      | 38.7 | 4,998        | 4,851          | –    | 28   |
| 7   | *B. drentensis* FJAT-10044 | LUUL00000000.1       | Scaffold      | 5.3       | 38.9 | 5,163        | 4,890          | 51   | 125  |
| 8   | *B. firmus* NBRC 15306     | BCUY00000000.1       | Contig        | 4.42      | 41.7 | 4,515        | 4,170          | –    | 26   |
| 9   | *B. firmus* NCTC10335      | UFTC00000000.1       | Contig        | 4.8       | 41.7 | 4,948        | 4,436          | 36   | 108  |
| 10  | *B. firmus* 14_TX          | QNSF00000000.1       | Scaffold      | 5.84      | 40.8 | 6,009        | 5,834          | 23   | 102  |
| 11  | *B. glycinifermentans* BGLY | LT603683.1          | Complete      | 4.61      | 46.1 | 4,735        | 4,381          | 24   | 82   |
| 12  | *B. glycinifermentans* SRCM103574 | CP035232.1     | Complete      | 4.81      | 46.0 | 4,948        | 4,572          | 25   | 82   |
| 13  | *B. licheniformis* ATCC 14580 | CP000002.3       | Complete      | 4.22      | 46.2 | 4,382        | 4,219          | 21   | 72   |
| 14  | *B. licheniformis* YNP1-TSU | NZ_CM007615.1       | Chromosome    | 4.25      | 45.9 | 4,388        | 4,227          | 4    | 66   |
| 15  | *B. mesorae* FJAT-13985    | LUUQ00000000.1       | Scaffold      | 5.81      | 40.3 | 5,553        | 5,309          | 40   | 104  |
| 16  | *B. mesorae* H20-5         | CP022572.1          | Complete      | 5.84      | 40.4 | 5,600        | 5,330          | 41   | 109  |
| 17  | *B. methanolicus* NGA3     | CP007739.1          | Complete      | 3.34      | 38.7 | 3,355        | 3,092          | 27   | 91   |
| 18  | *B. niacin* DSM 2923       | JRYQ00000000.1      | Scaffold      | 2.2       | 38.3 | 2,167        | 1,922          | 3    | 35   |
| 19  | *B. novals* NBRC 102450    | NZ_BCP00000000.1    | Contig        | 5.57      | 39.9 | 5,387        | 5,212          | –    | 31   |
| 20  | *B. novals* FJAT-14227     | LUUR00000000.1      | Scaffold      | 5.67      | 40   | 5,517        | 5,247          | 37   | 118  |
| 21  | *B. oceanisediminis* Bhandara28 | MBRK00000000.1   | Contig        | 5.88      | 40.8 | 5,925        | 5,705          | 33   | 106  |
| 22  | *B. soli* NBRC 102451      | BCVI00000000.1      | Contig        | 5.46      | 39.5 | 5,289        | 5,109          | 3    | 18   |
| 23  | *B. soli* DSM 15604        | NISV00000000.1      | Scaffold      | 5.58      | 39.7 | 5,451        | 5,148          | 43   | 92   |
| 24  | *B. subtilis* subsp. spizizenii str. W23 | CP002183.1    | Complete      | 4.03      | 43.9 | 4,116        | 3,912          | 24   | 77   |
| 25  | *B. subtilis* subsp. subtilis str. 168 | AL09126.3      | Complete      | 4.22      | 43.5 | 4,536        | 4,237          | 30   | 86   |
| 26  | *B. thuringiensis* serovar konkukian str. 97–27 | AE017355.1    | Complete      | 5.24      | 35.4 | 5,263        | 5,117          | 41   | 105  |
| 27  | *B. thuringiensis* YBT-1518 | CP005935.1         | Complete      | 6         | 35.4 | 6,371        | 5,837          | 45   | 90   |
| 28  | *B. vireti* LMG 21834      | ALAN00000000.1      | Contig        | 5.28      | 39.7 | 5,106        | 5,084          | –    | 21   |
| 29  | *B. vireti* DSM 15602      | LDNB00000000.1      | Scaffold      | 5.31      | 39.8 | 5,118        | 4,794          | 13   | 89   |
| 30  | *B. velezensis* FJAT-18017 | CP012602.1         | Complete      | 5.27      | 42.4 | 5,018        | 4,825          | 30   | 85   |
| 31  | *B. velezensis* FJAT-29814 | LMTJ00000000.1      | Scaffold      | 5.89      | 41.9 | 5,791        | 5,596          | 11   | 85   |
| 32  | *B. velezensis* X1          | CP008855.1         | Complete      | 3.42      | 38.1 | 3,433        | 3,103          | 36   | 122  |
| 33  | *B. velezensis* MUM 116    | MLYR00000000.1      | Contig        | 5.72      | 38.4 | 5,600        | 5,273          | 25   | 165  |
| 34  | *B. velezensis* OxB-1      | AP013294.1         | Complete      | 3.59      | 47.8 | 3,604        | 3,438          | 22   | 83   |
| 35  | *B. velezensis* WN066       | SMY00000000.1      | Contig        | 6.21      | 38.6 | 6,131        | 5,757          | 36   | 151  |
### Table 2: Statistical information of the 45 bacterial genomes used in this study (Continued)

| No. | Organism              | No. of NCBI accession | Level     | Size (Mb) | GC% | No. of Genes | No. of Proteins | rRNA | tRNA |
|-----|-----------------------|-----------------------|-----------|-----------|-----|--------------|----------------|------|------|
| 43  | Bacillus sp. 7884–1   | NPDD00000000.1        | Contig    | 6         | 38  | 5,848        | 5,597          | –    | 46   |
| 44  | Bacillus sp. MIRMR6   | MSL50000000.1         | Contig    | 5.44      | 38.8| 5,276        | 4,978          | 50   | 108  |
| 45  | Bacillus sp. S3       | CP039727.1            | Complete  | 5.58      | 40.3| 5,131        | 5344           | 104  | 36   |

*: unpublished

### Table 3: Genes associated with putative heavy metal (loid) resistance in Bacillus sp. S3

| Category                      | Gene ID     | Gene   | Protein                          | Function                                           |
|-------------------------------|-------------|--------|----------------------------------|---------------------------------------------------|
| Arsenate/arsenite detoxification | FAY30_05805 | aioB   | Small subunit of arsenite oxidase | As (III) oxidation                                 |
|                               | FAY30_02870 | arsB   | Putative arsenical pump membrane protein | As (III) efflux pump                               |
|                               | FAY30_09810 |        |                                  |                                                   |
|                               | FAY30_12790 |        |                                  |                                                   |
|                               | FAY30_09815 | arsC   | Arsenate reductase               | As(V) reduction                                    |
| Copper detoxification         | FAY30_07975 | copA   | Copper-exporting P-type ATPase; Lead, cadmium, zinc and mercury transporting ATPase | Cation translocation P-type ATPase |
|                               | FAY30_15235 |        |                                  |                                                   |
|                               | FAY30_16820 |        |                                  |                                                   |
|                               | FAY30_16835 |        |                                  |                                                   |
|                               | FAY30_11165 | copZ   | Copper chaperone                 |                                                   |
| Chromate detoxification       | FAY30_05265 | cutC   | Copper homeostasis protein       |                                                   |
|                               | FAY30_22440 | chrR   | Chromate reductase               |                                                   |
|                               | FAY30_11465 |        |                                  |                                                   |
|                               | FAY30_20185 | chrA   | Chromate transport protein       |                                                   |
|                               | FAY30_20190 |        |                                  |                                                   |
| Cadmium, zinc, cobalt, mercury detoxification | FAY30_09760 | cadC | Cadmium resistance transcriptional regulatory protein |                                                   |
|                               | FAY30_09765 | cadA   | Putative cadmium-transporting ATPase |                                                   |
|                               | FAY30_21075 | zupT   | Zinc transporter                 |                                                   |
|                               | FAY30_06055 | zosA   | Zinc-transporting ATPase         |                                                   |
|                               | FAY30_11960 | yeiR   | Zinc-binding GTPase              |                                                   |
|                               | FAY30_17115 | zur    | Zinc uptake regulation protein   |                                                   |
|                               | FAY30_15605 | znuA   | Zinc ABC transporter, substrate-binding protein |                                                   |
|                               | FAY30_20065 | znuB   | Zinc ABC transporter, permease protein |                                                   |
|                               | FAY30_08125 | znuC   | Zinc ABC transporter, ATP-binding protein |                                                   |
|                               | FAY30_20440 | czcD   | Cadmium, cobalt and zinc/H(+)-K(+)-antiporter | Cation efflux system protein |
|                               | FAY30_10430 | corC   | Magnesium and cobalt efflux protein |                                                   |
|                               | FAY30_24820 | corA   | Cobalt/magnesium transport protein |                                                   |
|                               |               | merR   | Mercuric resistance operon regulatory protein |                                                   |
| Nickel, molybdenum, detoxification | FAY30_20460 | nikMN | Nickel transport protein         |                                                   |
|                               | FAY30_20940 | modA   | Molybdate-binding periplasmic protein |                                                   |
|                               | FAY30_18400 | modB   | Molybdenum transport system permease protein |                                                   |
Core and pan genomes analyses

To clarify the genomic features specific to each Bacillus strain, all genes from tested Bacillus strains were described by MP method in the pan-genome analysis pipeline with a 50% cut-off for protein sequence identity. There were total 39,933 orthologous protein coding sequences in the pan genome for the Bacillus genus (Fig. 4a). Of these orthologs genes, 554 (1.38% of total pan genome) were identified as core conserved genes, and 16,234 were identified as strain-specific genes. The numbers of accessory genes varied from 1189 to 4431 (mean 3693) and the Bacillus sp. S3 had 3893 accessory genes. Accessory gene is known as indispensable orthologs, whose variability indicates the flexibility of genome structure [28]. After comparing strain-specific genes, the variability in the total number of strain-specific genes ranged from 0 to 1560 genes (mean 360). Bacillus sp. OxB-1 had the highest amount of these (n = 1560), reflecting the greatest difference with other tested genomes.

Previous reports stated that a mathematical extrapolation of the pan-genome was positively reliable when sufficient genomes (>5) were involved [29]. The deduced power law regression function \[ P_c(n) = 5313.92 n^{0.527477} \] revealed that the pan-genome of Bacillus had a parameter (\( \gamma \)) of 0.527477 (0 < \( \gamma \) < 1), implying a stabilized core structure and an open pan-genome of Bacillus strains (Fig. 4b). Thus, the new orthologs were intuitively observed after addition of more genomes to the group. Furthermore, the extrapolated curve of the core genome presented a steep slope according to the exponential regression \[ F_c(n) = 1779.45e^{-0.0387412 n} \] (Fig. 4b). The addition of an extra genome would not significantly alter the size of the core genome due to the numbers of core genes were relatively stable [25].

These core genes shared with all Bacillus genomes also could be classified into different COG categories (Fig. 4c), which was agreed with previous reports that larger prokaryotic genomes tended to pile up genes directly or indirectly involved in different metabolisms [30]. The KEGG annotation of 385 specific genes of Bacillus sp. S3 showed that 10 genes involved in the environmental information processing, including one cobalt transport system protein-encoding gene corA and one As (III) efflux pump membrane proteins-encoding gene arsB. Further, small numbers of strain-specific genes (< 40%) were assigned to the COG categories for the Bacillus sp. S3, which were mainly found to be enriched in phosphotransferase ABC-type metal ion transport systems. The result revealed specific adaptive strategies of Bacillus sp. S3 in response to harsh eco-environments.

Fig. 3 Phylogenetic relationships of 45 Bacillus strains. Phylogenetic trees based on (a) 16S rRNA genes derived from Bacillus sp. S3 and other closely-related strains, (b) 554 core genes. Bootstrap values are indicated at each node based on a total of 1000 bootstrap replicates. c Whole-genome-based phylogeny trees using a composition vector (CV) approach. Bacillus sp. S3 was marked in red blot and other strains that formed a group with Bacillus sp. S3 were marked in blue. Paenibacillus sp. Y412MC10 was used as an outgroup.
Mobile gene elements in *Bacillus* genomes

The presence of the majority of genomic islands (GEIs) in *Bacillus* sp. S3 and other comparative strains rendered clue about the genomic plasticity of these isolates (Table S5). We identified 20/5/15 GEIs in *Bacillus* sp. S3 through three methods. These GEIs were possibly conducive to the integrated pool of transposase. Analysis of transposable elements showed that numerous insertion sequences (IS) were distributed over the genomes and plasmids of *Bacillus* strains, harboring IS1, IS2, IS3, IS4, IS5, IS21 and IS256. The majority of IS could magnify the size of genome, and result in frequently genomic exchange with other community members [31]. As shown in Table S5, all *Bacillus* genomes could be served as feasible targets of phage infections. A total of 5 intact (100% score) prophage regions were predicted in the genome of *Bacillus* sp. S3, their information containing size: 11,485 bp, 5841 bp, 8630 bp, 8468 bp, 7959 bp; coding sequence: 11, 7, 6, 10, 8; GC content: 42.84, 38.02, 35.25, 36.25, 33.84% (Figure S6). Furthermore, the number of CRISPRs varied from 0 to 15 per strain and CRISPR locus absolutely scattered the chromosome. Four confirmed CRISPR loci were detected in *Bacillus* sp. S3 with 39, 20, 60, 4 spacers.

Comparative genomic analyses of *Bacillus* genus

Mauve has been used for constructing multiple genome alignments in large-scale evolutionary events such as genome rearrangement, inversion, and other recombination [32]. In order to prove the extent of genomic shuffling, the whole genome sequence of *Bacillus* sp. S3 was individually compared with the other 10 complete genomes using mauve with default settings. As shown in Fig. 5, synteny maps of the 10 complete genomes were inspected, and represented large-scale blocks of inversions and several crisscrossing locally collinear blocks (LCBs). 615 LCBs with a minimum weight of 45 were exhibited between *Bacillus* sp. S3 and other comparative strains rendered comparative information was also observed. Mauve analyses showed *Bacillus* sp. S3 had the highest synteny with *B. bataviensis* DSM21833, and other comparative information was also observed. Mauve analyses showed *Bacillus* sp. S3 had the highest synteny with *B. bataviensis* DSM21833 genome. Compared to *B. bataviensis* DSM21833, other strains exhibited more rearrangements, insertions and deletions. Conserved structural synteny and lack of inversions and rearrangements were observed among members of *Bacillus* group, suggesting that the large-scale evolutionary events were occurred at the genus

| Table 4: Average nucleotide identities (ANI) analysis of *Bacillus* sp. S3 and other *Bacillus* species |
|-----------------------------------------------|
|                | S3   | LMG 21833 | LMG 21834 | DSM 15602 | NBRC 102451 | NSP 15604 | FJAT-14227 | ATCC 14580 | YNP1-TSU | OM18 | LF1 | W23 | 168 | OK048 |
|----------------|------|-----------|-----------|-----------|-------------|-----------|------------|-----------|----------|------|-----|-----|-----|------|
| *B. bataviensis* | 81.34| *         | 78.7      | 78.4      | 78.4        | 79.2      | 99.99      | 79.8      | 66.8     | 69.62| 74.47| 76.72| 67.8 | 74.02|
| *B. vireti* LMG 21834 | 78.25| 78.78 | *         | 79.6      | 79.61      | 79.6      | 79.6       | 79.6      | 66.61    | 66.61| 66.61| 66.61| 66.61| 73.69|
| *B. vireti* DSM 15602 | 78.15| 78.7 | *         | 99.99     | 79.6      | 89.59     | 89.59      | 66.72     | 66.61    | 68.86| 74.48| 66.77| 68.68| 73.94|
| *B. soli* NBRC 102450 | 78.06| 78.73 | 79.74     | 79.74     | *          | 99.99     | 80.58      | 80.6      | 66.51    | 66.45| 69.01| 74.81| 66.75| 67.79| 74.28|
| *B. soli* DSM 15604 | 78.39| 79.05 | 79.94     | 79.92     | 99.99     | *          | 80.76      | 80.78     | 67.61    | 66.71| 69.99| 75.11| 67.9 | 67.92| 74.83|
| *B. novais* NBRC 102450 | 78  | 78.97 | 89.46    | 89.46     | 80.6      | 80.61     | *          | 100       | 66.7    | 66.6  | 68.54| 74.53| 66.69| 67.71| 74.08|
| *B. novais* FJAT-14227 | 78.42| 79.31 | 89.58    | 89.56     | 80.66     | 80.7      | 99.99     | *          | 67.63    | 67.04 | 69.49| 74.92| 67.55| 67.76| 74.43|
| *B. licheniformis* ATCC 14580 | 67.35| 67.09 | 67.24    | 67.29     | 67.08     | 67.16     | 67.32      | 67.33     | 99.57    | 66.99| 67.12| 72.15| 72.16| 67.13| 66.74|
| *B. licheniformis* YNP1-TSU | 66.76| 66.55 | 66.71    | 66.73     | 66.54     | 66.58     | 66.86      | 66.88     | 99.41    | 66.6  | 66.58| 71.83| 71.75| 66.74| 80.72| 70.21| 69.86| 69.8 | 70      | 70.05| 69.79| 69.85| 69.76| 69.79| 69.07| 68.52| 68.48| 68.93|
| *B. soli* | 74.3  | 74.32 | 74.64    | 74.66     | 75.01     | 75.04     | 74.75      | 74.78     | 66.63    | 66.64| 69.47 | 67.14| 67.19| 73.34|
| *B. subtilis* subsp. str. W23 | 67.61| 67.52 | 67.49    | 67.44     | 67.38     | 67.43     | 67.48      | 67.54     | 72.12    | 71.88| 67.83| 67.47 | 92.5 | 67.29|
| *B. subtilis* subsp. str. 168 | 67.67| 67.62 | 67.53    | 67.47     | 67.46     | 67.45     | 67.52      | 67.53     | 72.14    | 71.82| 67.87| 67.53 | 92.23| 67.57|
| *Bacillus* sp. OK048 | 74.04| 73.97 | 74.26    | 74.3      | 74.56     | 74.59     | 74.21      | 74.26     | 66.58    | 66.53| 69.16| 73.35| 66.86| 66.85| 74.02|

Numbers in green denote strains belonging to the same species. The asterisk indicates that the strain is compared to itself, which does not provide valuable information.
level [24]. This was in agreement with genome distance in phylogenetic tree.

The origin and evolution of Sb(III)-related genes

To explore the origin and evolution of Sb(III)-related genes in Bacillus sp. S3, typical genes including \( \text{aioB} \), \( \text{arsB} \), and \( \text{arsC} \) were selected for analysis. The deviant \( G + C \) contents between gene and the genome could be used as a detection method of HGT [27]. Herein, we detected the \( G + C \) contents of Sb(III)-related genes and their corresponding genomes in Bacillus strains. As shown in Figure S7, the average GC contents of the Sb(III)-related genes were different from that of their corresponding genomes in Bacillus strains. It is worth noting that the \( \text{aioB} \) gene was a specific gene of Bacillus sp. S3, whose GC content was significantly higher than that of the genome (44.64 vs. 40.3).

The overall origin and evolution of Sb(III)-related genes in Bacillus genus were inferred by phylogenetic trees with NJ, ML and UPGMA methods. We speculated that the \( \text{aioB} \) gene in Bacillus sp. S3 derived from the evolution of 2Fe-2S ferredoxin (Figures S8, S9 and S10). To further elucidate the evolution of \( \text{arsB}_{123} \) and \( \text{arsC} \), phylogenetic trees based on ArsB and ArsC proteins were constructed (Figures S11, S12, S13, S14, S15 and S16). As shown in Figure S11, S12, S13, the \( \text{arsB}_{123} \) genes of Bacillus sp. S3 formed separate 3 groups and a monophyletic clade, indicating that Bacillus sp. S3 might obtain \( \text{arsB}_{123} \) genes from \( B. \) bataviensis LMG21833, \( B. \) vireti and \( B. \) drentensis. Notably, the \( \text{arsB} \) gene of Bacillus cereus grouped with Klebsiella pneumoniae (Figure S11 and S12), the \( \text{arsB} \) gene of Bacillus megaterium grouped with Paenisporosarcina sp. HGH0030 (Figure S11 and S13). The results revealed that Bacillus species could acquire \( \text{arsB} \) gene from other genera via HGT. As shown in Figures S14, S15 and S16, the phylogenetic analysis revealed that the \( \text{arsC} \) gene of Bacillus sp. S3 only grouped with \( B. \) bataviensis, implying that \( B. \) bataviensis was likely donors of the \( \text{arsC} \) gene. Meanwhile, the \( \text{arsC} \) gene of Bacillus sp. 7884–1 grouped with Rhodococcus qingshengii, Bacillus oceanisediminis grouped with Xanthomonas citri (Figure S15), indicating that Sb(III)-related genes of Bacillus species might be acquired via HGT events from other genera. The results suggested that the \( \text{arsB} \) gene and \( \text{arsC} \) gene of Bacillus sp. S3 might originate from a common ancestor with \( B. \) bataviensis.

Assessment of functionality of heavy metal (loid)s-related genes

Previous reports showed that the CAI (codon adaptation index) was a numerical estimator of gene expression
level, due to highly expressed genes in bacteria were prone to magnify stronger codon bias [33]. The CAI value varies from 0 to 1.0, and higher CAI value indicates a higher expression level [34]. In order to indirectly assess the functionality of metal (loid) resistance genes, the appraisal of the strength of natural selection was performed, along with the CAI values of these genes. Putative highly expressed (PHX) genes associated to metal (loid) resistance in the Bacillus genus were inferred, where gerd gene encoding spore germination protein was used as a reference. As shown in Fig. 6a, the CAI values of these above-mentioned metal (loid) s resistance genes were calculated. The cutoff values were indicated with average CAI values of gerd genes in each species. Our result showed that only about 8% of the metal (loid) resistance genes were predicted to be PHX genes that greater than 0.75, while CAI values of lots of genes ranged from 0.4 to 0.8. The PHX genes lead to stronger codon bias than those of low expressed level genes due to codon translational selection.

A gene in the node or tip of a given tree was considered under diversifying selection (\(dN/dS > 1\)), evolving neutrally (\(dN/dS \approx 1\)) or purifying selection (\(dN/dS < 1\)) using the likelihood ratio test after adjusting for multiple testing (\(P\) value < 0.1) [33]. As shown in Fig. 6b, 96.3% of 10 selected genes associated metal (loid) had a ratio of

Fig. 5 Mauve alignment of Bacillus sp. S3 with its closer Bacillus genomes. Bacillus thuringiensis serovar konkukian str. 97–27 (a); Bacillus licheniformis ATCC 14580 (b); Bacillus glycinismentans BGLY (c); Bacillus velezensis FZB42 (d); Bacillus mesone H2O–5 (e); Bacillus batavensis LMG 21833 (f); Bacillus methanolicus MGA3 (g); Bacillus sp. Ox8–1 (h); Bacillus subtilis subsp. spizizenii str. W23 (i); Bacillus thuringiensis YBT-1518 (j). Boxes of different colors demonstrate the sequence coordinates and the conserved segments represented LCBs (or locally conserved regions). The LCBs above and below the reference line of the consistent color represent the orientation of the LCBs relative to the reference sequence for each genome. White areas represent possibly contain genome-specific sequence elements and those genomic positions that did not adequately align between the selected genomes.
nonsynonymous substitutions \((dN/dS < 1)\), implying that these genes were indispensable factor for the above-mentioned Bacillus species under the pressure of purifying selection. Only an \(\text{arsC}\) gene from \(B.\ bataviensis\) LMG21833 \((dN/dS = 1.63)\), double \(\text{chrA}\) genes from \(B.\ niacin\) DSM 2923 and \(B.\ liceniformis\) ATCC 14580 \((dN/dS = 2.08, dN/dS = 1.28)\), a \(\text{copZ}\) gene from \(B.\ firmus\) NCTC 10335 \((dN/dS = 1.28)\), a \(\text{corA}\) gene from \(Bacillus\ sp.\ LF1\) \((dN/dS = 2.80)\), double \(\text{znuB}\) genes from \(B.\ soli\) 15604 and \(B.\ bataviensis\) LMG21833 \((dN/dS = 2.02, dN/dS = 1.1)\) showed \(dN/dS > 1\), suggesting that they might be under diversifying selection. Furthermore, the lowest \(dN/dS\) ratio was remarked for \(\text{copA}\) gene \((\text{average } dN/dS = 0.08)\) and the \(\text{zur}\) gene \((\text{average } dN/dS = 0.09)\), demonstrating strong purifying selection. Additionally, these genes including \(\text{arsB}\) from \(Bacillus\ mesonae\) H20–5, \(\text{chrR}\) from \(Bacillus\ mesonae\) H20–5 and \(Bacillus\ sp.\ S3\), \(\text{copZ}\) from \(B.\ thuringiensis\) 97–27, \(Bacillus\ glycinifermentans\) BGLY and \(B.\ oceanisediminis\) Bhandara28, \(\text{corA}\) from \(B.\ firmus\) 14_TX, showed \(dN/dS\) ratio = 1.0, indicating that selection force had little effect on them. The results showed that metal (loid) resistance genes had universally low \(dN/dS\) ratios and high CAI values, indicating that their functions played a role in supporting the growth of Bacillus species in response to harsh environments [34].

Transcriptional expression analysis in Bacillus sp. S3 with or without Sb(III)
To gain the insights into the role of Sb(III)-related genes, the expression levels of \(\text{aioB}\), \(\text{arsB}_{123}\), \(\text{arsC}\) and \(\text{psts}_{1}\) were investigated by RT-qPCR. Primers used in the study were listed in Table S6, where 16S rRNA gene was used as an internal reference. As shown in Fig. 7, the transcriptional expression levels of most of genes were up-regulated by Sb(III) except for \(\text{arsC}\). Although the expression levels of \(\text{aioB}\) and \(\text{arsB}_{123}\) in exposure of 0.5 h Sb(III) were down-regulated, the expression levels were up-regulated after 1 h, 2 h and 4 h, respectively. Compared to uninduced culture, the expression level of \(\text{aioB}\) gene increased 15.8, 4.4 and 2.6 folds with 100 \(\mu\text{M}\) Sb(III) from 1 to 4 h. Nevertheless, when the Bacillus sp. S3 was exposed to high Sb(III) concentration \((200\text{ and }300\text{ }\mu\text{M})\), the expression level of \(\text{aioB}\) gene was up-regulated 2.6, 2.1, 1.3 and 2.5, 2.8, 2.1 folds from 1 to 4 h, respectively. Thus, the expression level of \(\text{aioB}\) was independent of Sb(III) concentration and stress time. As shown in Fig. 7, the expression levels of \(\text{arsB}_{123}\) (2 h and 4 h) were remarkably up-regulated. The expression levels of \(\text{arsB}_{123}\) and \(\text{psts}_{1}\) were up-regulated along with the increase of Sb(III) concentration. The increased expression levels of \(\text{aioB}\) and \(\text{arsB}_{123}\) in presence of Sb(III) suggested that Sb(III) could stimulate the expression levels of As (III)/Sb(III) resistance genes, which might act synergistically to release the toxicity of Sb(III) in Bacillus sp. S3.

Discussion
In the previous study, Bacillus sp. S3 showed high Sb(III) oxidation activity and multiple heavy metal (loid) s resistance capability [18]. The morphological characterization of Bacillus sp. S3 in presence of heavy metal (loid) s was performed in this work. The SEM results showed that the elevated levels of heavy metal (loid) ions might suppress the secretion of extracellular polymers substance and normal metabolism (Fig. 1). The smallness of bacterial cells provided a large contact interface, which would facilitate the interaction between heavy metal (loid) s and biosorption process of Bacillus sp. S3. The EDS spectra results revealed that Bacillus sp. S3 might further absorb Pb (II) compared with other

![Fig. 6 Ranges of CAI values of different metal resistance genes of Bacillus species with getD gene as a reference (a). Distributions and ranges of selection pressure on various metal resistance genes within the Bacillus genus (b)](image)
heavy metal (loid) s, such as extracellular adsorption and surface complexation [9].

Sb-oxidizing bacteria can convert Sb(III) to the less toxic Sb(V), which is very important for environmental Sb bioremediation [14]. In this study, the newly sequenced Bacillus sp. S3 represented a complete genome, including a circular chromosome and a circular plasmid. To understand the molecular mechanisms that Sb(III) oxidation and resistance, a series of Sb(III)-related genes in Bacillus sp. S3 genome were mined, such as aioB, arsB, arsC and Pi-related genes. The aioAB genes encoding As (III) oxidase were responsible for Sb(III)/As (III) oxidation, which could convert the more toxic Sb(III) to the less toxic Sb(V) in the periplasm [12]. The data we presence demonstrated a first glimpse into the aioB gene encoding arsenate oxidase in Bacillus genus. These Sb(III)-related genes of Bacillus sp. S3 might play a key role in coping with Sb(III)-polluted sites. Recently, the cytoplasmic Sb(III) oxidase AnoA was identified and characterized in A. tumefaciens GW4 by comparative proteomics and RT-qPCR [22]. Compared to A. tumefaciens GW4, Bacillus sp. S3 has remarkably higher Sb(III) resistance and Sb(III) oxidation ability. However, the anoA gene was not found in Bacillus sp. S3, indicating other unknown mechanisms. Of course, the genome of Bacillus sp. S3 was also harbored a high numbers of other heavy metal (loid) s resistance genes, since many contaminated sites contain multiple heavy metal (loid) s [16]. These results revealed various adaptative mechanisms of Bacillus sp. S3 to survive in metal-contaminated environments. It has been reported that resistance genes in response to nitrate and heavy metals were propelled by harsh eco-

Fig. 7 Real-time quantitative PCR analysis of the genes encoding proteins involved in antimonite/arsenate oxidation (a), antimonite/arsenite resistance (b, c, d and e) and phosphate metabolism (f). Data shown as the mean of three replicates with the error bars representing ± SD.
environments, which involved in defense and repair mechanisms for dealing with heavy metals [10].

Although 16S rRNA gene has been conventionally used for assessing bacterial taxonomy and phylogeny, there were still controversy and uncertainty based on only one gene [35]. To implement the taxonomic classification of Bacillus sp. S3 in Bacillus genus, the phylogenetic trees based on 554 core genes and whole-genome were constructed. The phylogenetic trees based on 554 core genes and whole-genome showed that Bacillus sp. S3 was closest to the Bacillus bataviensis. To confirm the findings from the phylogenetic analysis, the ANI and dDDH% analyses were performed. ANI is the most widely accepted bioinformatics tool that evaluate the genomic distance and delineate species in evolutionary progress, overcoming the difficulty of conventional deviations caused by evolutionary mutation rate and HGT events [36]. Besides, the value of 70% dDDH was the recommended standard for species delineation of bacteria, corresponding tightly to 95% ANI [37]. The results of ANI and dDDH values indicated that Bacillus sp. S3 represented a novel species.

It is well recognized that bacteria genomes have notable genome plasticity by several elements of HGT events, known as mobile gene elements (MGEs, IS, Prophages and CRISPRs) and plasmids [31]. The results showed that the majority of MGEs were distributed on chromosomes within the Bacillus genus. MGEs were key driving forces of genome evolution and played a pivotal role in HGT events [11], indicating that high genomic plasticity in Bacillus genus was extended to potential strategies to cope with high metal (loid) ion concentrations of their natural habitats. GEIs, which have been committed to provide antibiotic resistance to the host bacteria, were generally divided into 4 categories based on their function, including resistance island (RIs), virulence genes, metabolic islands, and symbiotic island (SIs) [38]. These islands promoted symbiotic integration of the host with other microorganisms [39]. CRISPR-Cas system is a type of adaptive immunity in bacteria and archaea, which protect them against invading genetic elements [40]. Our findings suggested that the Bacillus genus could trigger various defense mechanisms against the invasion of exogenous DNA for maintaining the stability of their genetic architecture during the evolution.

Genome evolution could be driven by the acquisition and loss of genes, which was conducted by HGT, genomic reshuffling and natural selection [41]. A combination of several approaches was implemented to discover putative HGT, since it was difficult to identify via either phylogenetic analysis or deviant G + C content [5]. Our results suggested that the prosperus branch of the arsB_123 genes of Bacillus sp. S3 near the base of the B. bataviensis LMG21833, B. vireti and B. drentensis, and the arsC gene grouped with B. bataviensis. The results suggested that the arsB_123 genes and arsC gene in Bacillus sp. S3 were acquired via HGT from other Bacillus species, including B. bataviensis, B. vireti and B. drentensis. Such results were found to be consistent with a previous study which examined evolution of ars genes in Pantoea spp., in which bacterial genes related to As resistance and detoxification might be acquired via HGT [42]. A previous study showed that a high numbers of metal resistance genes of C. testosteroni S44 shared highest similarities with C. testosterone KF-1 or C. testosterone CNB-2, but not with other genera [16]. However, the arsC gene of Bacillus sp. 7884–1 might be acquired via HGT events from Rhodococcus qingshengii (Figure S15), and the arsB gene of Bacillus cereus might be acquired via HGT events from Klebsiella pneumoniae (Figure S11). The results suggested that Sb(III)-related genes might be acquired from other genera during evolution of the Bacillus genus.

Transcriptional patterns of the different Sb(III)-related genes were quite different, indicating the various response of Bacillus sp. S3 to Sb(III) detoxification. In the case of Sb(III)-related genes, aioB, arsB_123 and psts_1 were up-regulated by Sb(III), while the arsC gene was down-regulated by Sb(III). On the contrary, with the addition of Sb(III), the arsC1 and arsC2 genes showed 2.9 and 4.7 folds up-regulation in Agrobacterium tumefaciens, respectively [22]. It has been reported that aioA expression was not induced by Sb(III) in A. tumefaciens GW4 [11]. In contrast, our results showed that the expression level of aioB was up-regulated from 1 to 4 h compared to uninduced cell, indicating that the aioB gene was induced by Sb(III) and played a putative role in oxidizing Sb(III). Nevertheless, 0.5 h Sb(III) exposure showed that the expression level of the aioB gene was down-regulated during the earlier time points. It was noteworthy that the expression level of the aioB gene in higher Sb(III) concentration (200 and 300 μM) notably lower than 100 μM Sb(III), indicating that the higher Sb(III) concentration could inhibit aioB expression. These results were basically consistent with the previous reports that the excessive As (III) treatment could inhibit the aioAB expression [12]. In addition, the expression level of psts_1 gene involved in phosphate metabolism and co-regulated the aioAB genes was induced by Sb(III), suggesting that Bacillus sp. S3 required the DNA repair and amino acids synthesis processes in response to Sb(III) by enhancing production of Pi and phosphoribosyl pyrophosphate [22].

Conclusion

In this study, we sequenced a hyper Sb(III) oxidation strain Bacillus sp. S3 and performed comparative genetic study of the Bacillus group, representing
substantial improvements over previously published results. The majority of genes encoding metal (loid) resistance proteins and MGEs were discovered in *Bacillus* sp. S3, which could adapt to metal (loid)-contaminated environments. Meanwhile, there was an arsenate oxidase AioB in the *Bacillus* sp. S3, which could play a key role in the process of Sb(III)-oxidizing. Notably, *Bacillus* sp. S3 was identified as a new species by phylogenetic trees and ANI analysis. The origin and evolution analysis of Sb(III)-resistance genes was carried out. In addition, Sb(III)-related genes in the *Bacillus* sp. S3 were induced by Sb(III) using RT-qPCR, indicating these genes occupied a significant position in alleviating the toxicity of Sb(III). These findings could improve our understanding of the genomic characteristics and evolutionary relationships among the *Bacillus* genus. As a consequence of the lack of comprehensive analysis with respect to genetic expression and regulation by *Bacillus* sp. S3, the molecular basis of microorganism-Sb(III) needs to be further elucidated in the near future through gene knockout and protein characterization.

**Methods**

**Bacterial strain and culture conditions**

*Bacillus* sp. S3 was previously isolated from an antimony-mine area, in Hunan province, China [17]. The bacterial cells of *Bacillus* sp. S3 were aerobically grown in 50 ml. Luria broth (LB) medium (10.0 g/L tryptone, 5.0 g/L yeast extract, and 5.0 g/L NaCl, pH 7.0–7.2) with shaking at 150 rpm at 28 ± 2 °C. The bacterial growth was measured using ultraviolet-visible spectrophotometer (Shimadzu UV-2550, Japan) by recording optical density at 600 nm (OD 600). To determine the hazardous effects of different heavy metal (loid) s on the bacterial growth, different concentrations of C8H4K2O12Sb2·3H2O, NaAsO2, CdCl2, K2CrO4, PbNO3, Cu(SO)4, ZnCl2 was added to the culture aliquots [18]. The total concentrations of Sb(III), As (III), Cd (II), Cr (VI), Pb (II), Cu (II) and Zn (II) in each treatment were 100, 1000, 50, 200, 500, 800 and 80 μM, respectively.

**Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analysis**

Bacterial cells with different metal treatments were firstly harvested in the exponential phase at 4 °C at 4000×g for 10 min when the OD600 reached 0.8, which were then fixed at 4 °C in 2.5% glutaraldehyde for about 2  h. After being washed twice with phosphate buffer saline (PBS, 0.1 M and pH 7.0) and dehydrated by ethanol (30–100%) for 20 min, the cells samples were dehydrated using a lyophilizer and coated with a thin layer of platinum via vapor deposition. The surface morphology and property of the *Bacillus* sp. S3 cell were analyzed using a SEM (Helios NanoLab G3 UC, Thermo Fisher Scientific, Czech; accelerating voltage: 15 kV) equipped with an energy dispersive X-ray analyzer (X-stream-2; Oxford instruments, Oxford, UK).

**Whole-genome sequencing, assembly and annotation**

The genomic DNA of *Bacillus* sp. S3 was extracted using an E.Z.B.A Bacterial DNA Kit (Omega, Bio-Tek, USA) according to the manufacturer’s instructions. The strategy of whole genome sequencing was used a combination of Illumina HiseqXten (Illumina Inc., San Diego, CA, USA) and Pacific Biosciences Sequel sequencing platform (Pacific Biosciences, Menlo Park, CA, USA), and the PE sequence data from the Illumina platform was performed by proofread PacBio assembly sequence. Illumina libraries were prepared using NEXTflexTM Rapid DNA-seq Kit (BIOO Scientific Crop., Austin, TX, USA) in terms of the included instructions. A 10-kb SMRT bell library was prepared from sheared genomic DNA using a 10-kb template barcoded library preparation workflow. Single Molecule Real Time (SMRT) sequencing was conducted on a PacBio Sequel sequencing platform using the SMRT v3.0 cell. For the *Bacillus* sp. S3 strain, a total of 88,313 clean reads with an average length of 9904 bp and an N50 size of 13,621 bp were generated (Table S7, S8, and S9).

De novo assembly of the PacBio read sequences was performed using continuous long reads (CLR) following the Hierarchical Genome Assembly Process (HGAP4) workflow (PacBio DevNet; Pacific Biosciences) as available in SMRT Link [43]. HGAP 4 consists of preassembly, de novo assembly with Celera Assembler, and assembly polishing with Quiver. To improve the accuracy of the assembly genome, four rounds of iterative error correction were carried out using the Illumina clean data by in house script. The final assembly generated a circular genome sequence with gapless. The circular maps of chromosome and plasmid were generated using the Circos software [44]. The genome and plasmid sequences of *Bacillus* sp. S3 were deposited in the NCBI database under the accession numbers CP039727.1 and CP039728.1, respectively.

Protein-coding regions in the assembled sequences were predicted using Prodigal [45]. tRNA and rRNA were separately identified by tRNA-scan and RNAmmer, respectively [45]. Genome annotation was carried out by a command line software tool: rapid prokaryotic genome annotation (Prokka) [46]. The genes functions were determined against the NCBI non-redundant (NR), Gene Ontology (GO), Clusters of Orthologous Groups (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases with E-value cut-off set to 1e−5 and subsequent filtering for the best hit [47].
Phylogenetic analysis and average nucleotide identity (ANI)
To perform the comparative analysis, 44 most closely related species and representative species were retrieved from the GenBank database by BLASTP search (Table 2). Three different datasets of representative markers, 16S rRNA sequences, core genes and whole genomes were used to construct phylogenetic trees. We obtained the 16S rRNA gene sequences of some strains closely related to Bacillus sp. S3 by BLASTP search against the NCBI database. The phylogenetic tree of Bacillus sp. S3 based on the 16S rRNA gene sequences was constructed using the NJ method in MEGA v7.0 with 1000 bootstraps replications [48]. Phylogeny based on only one common gene may result in bias; therefore, we constructed the phylogenetic tree based on the core genes, which were shared by the comparative strains (see ‘Comparative genomics analysis’). The core genes of the 44 Bacillus strains were fetched by Bacterial Pan Genome Analysis (BPGA) software [49]. The multiple sequences alignments of copy core genes was performed using MUSCLE software [50] and the phylogenetic tree was generated using the NJ method in MEGA v7.0 with 1000 bootstraps replications. In addition, the phylogenetic tree based on whole-genomes of Bacillus strains was also constructed in our study, in which Paenibacillus sp. S3 genome and the Paenibacillus sp. S3 strain was designed as an outgroup [51]. The average nucleotide identity (ANI) values between the newly sequenced Bacillus sp. S3 genomes and the representative genomes of Bacillus spp. were calculated using the web server JSpecies1.2.1 [32] based on a BLAST algorithm and tetranucleotide frequency correlation coefficient (Tetra) with default parameters [52]. In addition, the DNA-DNA hybridization (DDH) values were calculated using Genome-to-Genome Distance Calculator (GGDC) [53].

Comparative genomics analysis
BPGA was used to extrapolate pipeline pan-genome models with default parameters, and all of orthologous groups among testing Bacillus genus were identified [49]. The core genome is the common set of shared genes among all testing strains, the pan genome is the entire set of genes within test genomes, the accessory genome is the set of genes shared with more than two but not all testing strains and unique genes is the set of genes in each strain not shared with other strains [47]. The details of the strains used were listed in Table 2. Furthermore, synteny maps were generated to unravel the degree of rearrangements (insertions, deletions, duplications) by identifying conserved LCBs among genomes. Multiple alignments of Bacillus sp. S3 and 10 selected Bacillus genomes were also performed using the Mauve Genome Alignment v2.3.1 with the progressive Mauve algorithm [54].

Genes for heavy metal (loid) s resistance
The genes related to the resistance of Sb(III) and other heavy metal (loid) s in the Bacillus sp. S3 genome and other comparative Bacillus genomes were identified by performing BLASTP searches against the BacMet database [55]. Subsequently, each Sb(III) resistance gene was compared on NCBI database to find these genes of high similarity. Finally, the evolutionary relationships of genes related to Sb(III) was inferred using the NJ, maximum likelihood (ML), and UPGMA methods in MEGA v7.0 with 1000 bootstraps [5].

Prediction of mobile genetic elements (MGEs)
GEIs were detected using the web server IslandViewer4 with three prediction methods, including IslandPath-DIMOB, SIGI-HMM, and IslandPick with default parameters [56]. Insertion sequences were predicted and classified using the ISFinder platform against the ISfinder database with default criteria [57]. CRISPR arrays were detected using the CRISPR Finder online server to perform BLAST searches against dbCRISPR (CRISPR database) [58]. PHAST was used to scan prophages by BLASTing against the NCBI and the prophage databases [59].

Selective pressure analysis and expressivity prediction
The CAI values of selected genes were analyzed using Codon W1.4.2 (http://codonw.sourceforge.net//) and CAI Calculator 2 (http://www.evolvingcode.net/codon/CalculateCAIs.php). The mode and strength of natural selection in protein sequences were estimated by evaluating the ratio of nonsynonymous (dN) to synonymous (dS) nucleotide substitutions rates using the online software Datamonkey, and the HyPhy package with Single-Likelihood Ancestor Counting method was used to detect the selection sites [60].

Real-time quantitative PCR (RT-qPCR)
When Bacillus sp. S3 growth reached middle exponential phase, culture aliquots were amended with 0, 100, 200 and 300 μM of Sb(III), respectively. The culture aliquots were withdrawn at different time intervals (0.5, 1, 2 and 4 h) and cells were harvested by centrifugation at 4000×g for 10 min at 4 °C. After quick freezing in liquid nitrogen, the total RNA of each sample was isolated and purified using the E.Z.B.A Bacterial RNA Kit (Omega, Bio-Tek, USA) according to the manufacturer’s instructions. The concentration of RNA samples was measured at the A260/A280 ratio using a NanoDrop ND-1000 Spectrophotometer (BioTek Instruments, Inc., Vermont, USA) and the integrity of the RNA samples was
Statistical analysis
All experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). Statistical analyses were carried out by one-way ANOVA with post-hoc test. Tukey’s test (p < 0.05) in SPSS version 21.0 (SPSS Inc., Chicago, IL, USA).

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-020-01737-3.

Additional file 1: Figure S1. Energy dispersive X-ray spectroscopy (EDS) analysis of Bacillus sp. S3 after the different heavy metal ions exposure: (A) Sb(III); (B) As (III); (C) Cd (II); (D) Cr (VI); (E) Pb (II); (F) Cu (II). Figure S2. COG classification statistics of the Bacillus sp. S3 genome annotation. Figure S3. GO classification statistics of the Bacillus sp. S3 genome annotation. Figure S5. Distribution of CAZymes in Bacillus sp. S3. Figure S6. Gene contents of the intra-prophages in Bacillus sp. S3 predicted by PHAST. Figure S7. Comparison of G + C contents of these functional genes with those of the average of the entire genomes. (A) arsB_1; (B) arsB_2; (C) arsB_3; (D)OlarC. Figure S8. Neighbor-joining phylogenetic tree of concatenated AioB protein sequences derived from Bacillus sp. S3 and other representative species. Bacillus sp. S3 was marked in red blot. Figure S9. Maximum likelihood phylogenetic tree of concatenated AioB protein sequences derived from Bacillus sp. S3 and other representative species. Bacillus sp. S3 was marked in red blot. Figure S10. Phylogenetic tree analysis based on concatenated AioB protein sequences using UPGMA method under p-distance model. Bootstrap values were indicated at each node based on a total of 1000 bootstrap replicates. Bacillus sp. S3 was marked in red blot. Figure S11. Neighbor-joining phylogenetic tree of concatenated ArsB protein sequences derived from Bacillus sp. S3 and other representative species. Bacillus sp. S3 was marked in red blot. Figure S12. Maximum likelihood phylogenetic tree of concatenated ArsB protein sequences derived from Bacillus sp. S3 and other representative species. Bacillus sp. S3 was marked in red blot. Figure S13. Phylogenetic tree analysis based on concatenated ArsB protein sequences using UPGMA method under p-distance model. Bootstrap values were indicated at each node based on a total of 1000 bootstrap replicates. Bacillus sp. S3 was marked in red blot. Figure S14. Neighbor-joining phylogenetic tree of concatenated ArsC protein sequences derived from Bacillus sp. S3 and other representative species. Bacillus sp. S3 was marked in red blot. Figure S15. Maximum likelihood phylogenetic tree of concatenated ArsC protein sequences derived from Bacillus sp. S3 and other representative species. Bacillus sp. S3 was marked in red blot. Figure S16. Phylogenetic tree analysis based on concatenated ArsC protein sequences using UPGMA method under p-distance model. Bootstrap values were indicated at each node based on a total of 1000 bootstrap replicates. Bacillus sp. S3 was marked in red blot.

Table S1. COG functional categories of Bacillus sp. S3. Table S2. GO categories of Bacillus sp. S3. Table S3. KEGG categories of Bacillus sp. S3. Table S4. Digital DNA-DNA hybridization (dDDH) values between Bacillus sp. S3 and other Bacillus genomes. Formula I, II and III represented different methods used by GGDC to calculate the similarities. Table S5. Mobile genetic elements predicted in Bacillus genomes by different methods. Table S6. Primers used in RT-qPCR. Table S7. The quality control of clean data. Table S8. The statistical information and of clean data. Table S9. The statistical information of genome sequencing and assembling procedures.

Abbreviations
ANI: Average nucleotide identity; BLAST: Basic local alignment search tool; CAI: Codon adaptation index; CDSs: Protein-coding genes; dDDH: digital DNA-DNA hybridization; GEIs: Genomic island; IS: Insertion sequences; LCB: Locally collinear block; MGE: Mobile genetic element; ML: Maximum likelihood; NCBi: National center for biotechnology information; NJ: Neighbor-joining; RT-qPCR: Real-time quantitative polymerase chain reaction

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Authors’ contributions
GT performed the experiments and wrote the manuscript. LJ, QG, and ZW conceived the project and provided funds. LL, WX and LJ designed the experiments and analyzed the sequence data. SL, LY and YR assisted in running some of the data analysis and revising the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
The genome and plasmid sequences of Bacillus sp. S3 were deposited in the NCBI database under the accession numbers CP039727.1 and CP039728.1, respectively. All sequences involved in this study are available from the NCBI database.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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