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Arsenite Oxidation in *Ancylobacter dichloromethanicus* As3-1b Strain: Detection of Genes Involved in Arsenite Oxidation and CO_2 Fixation

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Abstract The aim of this study was to characterize a facultative chemolithotrophic arsenite-oxidizing bacterium by evaluating the growth and the rate of arsenite oxidation and to investigate the genetic determinants for arsenic resistance and CO_2 fixation. The strain under study, *Ancylobacter dichloromethanicus* As3-1b, in a minimal medium containing 3 mM of arsenite as electron donor and 6 mM of CO_2–bicarbonate as the C source, has a doubling time (t_d) of 8.1 h. Growth and arsenite oxidation were significantly enhanced by the presence of 0.01 % yeast extract, decreasing the t_d to 4.3 h. The strain carried arsenite oxidase (*aioA*) gene highly similar to those of previously reported arsenite-oxidizing Alpha-proteobacteria. The RuBisCO Type-I (*cbbL*) gene was amplified and sequenced too, underscoring the ability of As3-1b to carry out autotrophic As(III) oxidation. The results suggest that *A. dichloromethanicus* As3-1b can be a good candidate for the oxidation of arsenite in polluted waters or groundwaters.

Introduction

Arsenic is found in many environments and is toxic to life when present in the inorganic soluble form as arsenite, As(III), and arsenate, As(V). The various strategies developed by bacteria to transform arsenic include arsenite oxidation, cytoplasmic arsenate reduction, respiratory arsenate reduction, and arsenite methylation [23, 27]. Arsenite-oxidizing bacteria oxidize As(III) to As(V) by a detoxification mechanism because As(V) is much less toxic than As(III). The arsenite-oxidizing bacteria isolated so far are phylogenetically diverse. Arsenite-oxidizing bacteria oxidize As(III) to As(V) by the action of the periplasmic arsenite oxidase. The enzyme contains two subunits: a small Fe–S Rieske subunit and a large Mo-pterin subunit [28]. Recently, nomenclature for genes involved in prokaryotic aerobic arsenite oxidation was unified and the name assigned to the operon is *aio* [17]. Specifically, the new gene name for As(III) oxidase is *aioA*. Arsenite oxidase like-genes have been successfully amplified from a variety of soil, sediment, and geothermal environments [5, 14, 15, 19, 21, 24]. For numerous bacterial strains (heterotrophic arsenite-oxidizers), the oxidation of As(III) is considered a detoxification mechanism, even if in these bacteria, As(III) may be used as a supplemental energy source [3]. In contrast, certain strains are able to use arsenite as the source of energy and reducing power (chemolithotrophic arsenite-oxidizers) to grow in the presence of carbon dioxide under both aerobic [4, 9, 10, 25] and nitrate-reducing [20] conditions.

CO_2 fixation in chemolithotrophic bacteria generally occurs through the Calvin–Benson–Bassham cycle mediated by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which occurs in ecologically and evolutionary diverse organisms from all domains of life. Proteobacteria contain RuBisCO Types- I and -II, whose large subunits are encoded by the *cbbL* and *cbbM* genes, respectively [29]. Type-I is active in plants, green algae, cyanobacteria, and most eubacteria [1], whereas Type-II represents ancient enzymes mainly found in anaerobic environments [2].

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Ancylobacter dichloromethanicus As 3-1b is a Proteobacterium previously isolated from a rhizospheric agricultural soil (Toscana, Italy) polluted with 250 mg kg^{-1} of total As deriving from mining wastes containing arsenopyrite. The strain oxidizes As(III) in batch cultures under heterotrophic conditions [6]. In this study, we evidenced the facultative chemolithotrophic growth of the strain on As(III). *AioA* gene for As(III) oxidase has been identified in the isolate. The chemolithotrophic nature of the strain was supported by the positive amplification of the *cbbL* gene for RuBisCO Type-I.

Materials and Methods

Growth Characteristics of As3-1b

Strain As3-1b was maintained in glycerol stocks at −70 °C. Before use, the cells were grown to mid-exponential phase in a defined mineral medium containing a vitamin solution (BBM) and supplemented with Na gluconate (0.6 %, w/v) as C source (BBMG) at 28 °C [6].

Phenotypic data of As3-1b were determined with API 20E and 20NE identification systems (BioMerieux, France). Growth was scored after 24 and 48 h of incubation at 28 °C. The strain was tested on two separate occasions, and the agreement was very good.

Arsenite Oxidation Analysis

As(III) oxidation was studied under chemolithotrophic and mixotrophic conditions. At the purpose, cells of As3-1b were grown in BBM medium supplemented with 6 mM $NaHCO_3$ (BBMC) with and without As(III) in the following conditions: (i) BBMC medium prepared with HPLC-grade water, (ii) BBMC medium prepared with HPLC-grade water containing 0.01 % (w/v) yeast extract (YE), and (iii) BBMC medium prepared with HPLC-grade water and lacking vitamin solution. To prepare the inoculum, the strain was grown overnight in BBMG medium containing 3 mM of As(III), pH 8.0. Afterward, the cells were harvested, washed three times with sterile water to eliminate traces of organic C, and then suspended in sterile (0.2-μm filter) physiological saline solution to give an OD_{620} of 0.132, corresponding to 1.5×10^8 cells ml^{-1}. Three flasks for each condition were inoculated with the cell suspension (5 % v/v) and then incubated at 28 °C under agitation at 250 rpm on a rotary shaker. Three control flasks without inoculum were incubated to check for abiotic transformation of As. At different sampling times, 2-ml aliquots of the growing cultures were removed to measure the cell growth and the concentrations of As(III) and As(V). Cell growth was estimated by counting the total bacterial numbers using 4′,6-diamidine-2-phenylindole (DAPI) stain [16]. The number of bacteria was determined by counting the cells in 20–30 microscopic fields (at ×1,000 magnification) using an eyepiece with a calibrated reticule. The counts were expressed as Log of cell numbers ml^{-1}. The concentrations of As(III) and As(V) were spectrophotometrically determined according to the procedure proposed by Dhar et al. [7]. Standards were prepared for concentrations ranging from 0 to 1 M for both As(V) and As(III) from $Na_2HAsO_4 \cdot 7H_2O$ and $NaAsO_2$ (Sigma-Aldrich) solutions, respectively.

Detection and Phylogeny of As(III) Oxidase and RuBisCO Genes

Amplification of As(III) oxidase gene (*aio*A) was conducted with primers aoxBM1-2F (5′-CCACTTCTGCATCGTGGGNTGYGGNTA-3′) and aoxBM3-2R (5′-TGTCGTTGCCCCAGATGADNCCYTTYTC-3′) according to the protocol of Quèmèneur et al. [22]. For the amplification of the gene coding for the large subunit of RuBisCO Type-I (*cbbL*), the primers RBCO-1Cf (5′-GAACATCAAYTCKCAGCCCTT-3′) and RBCO-1Cr (5′-TGGTGCATCTGVCCGGCRTG-3′) were used according to the procedures of Alfreid et al. [2]. Detection of *cbbM* gene encoding for the large subunit of RuBisCO Type-II was performed with primers 168f RBCO-I (5′-CGGCACSTGGACCACSGTSTGGAC-3′) and 766r RBCO-I (5′-GTARTCGTGCATGATGATSGG-3′) according to Alfreid et al. [1]. The degenerate nucleotide sites are indicated by standard ambiguity codes as follows: N = A, C, G, or T; R = A or G; V = A, C, or G; and Y = C or T. All the reagents were obtained from Invitrogen. The PCR reactions were carried out using the T-Gradient Biometra apparatus. The PCR products were checked on a 2 % agarose gel and visualized by ethidium bromide staining using the Gel Doc image analyzer system.

The amino acid sequences deduced from the *aioA* and *cbbL* nucleotide sequences were compared with the entire collection of GenBank database using the BlastX program (http://www.ncbi.nlm.nih.gov/Blast/). Phylogenetic analysis of the deduced aminoacid sequences of *aioA* and *cbbL* genes was carried out using the MEGA version 4.0 software [30]. The *aioA* and *cbbL* sequences obtained in this study were deposited under the accession numbers FR747823, FR747824, and FR747825.

Results

Characterization of *A. dichloromethanicus* As3-1b

The strain was a Gram-negative non-spore-forming rod-shaped catalase-positive motile bacterium. The biochemical

and physiological characteristics listed in Table 1 evidenced that the strain is able to assimilate a great variety of sugars and organic acids as the sole carbon source and to ferment glucose, arabinose, mannitol, melibiose, inositol, rhamnose, sorbitol, sucrose, and amygdalin.

Chemolithotrophic and Mixotrophic As(III) Oxidation

The growth of As3-1b and the speciation of arsenic in lithotrophic and mixotrophic conditions were monitored.

As(III) oxidation and cell growth curves of strain As3-1b in BBMC medium with As(III) as the electron donor and CO_2–HCO_3^- as the C source are shown in Fig. 1a. Strain As3-1b behaved as autotrophic As(III)-oxidizer and gained energy from As(III) oxidation. The weak growth in the BBMC medium without As(III) may be linked to the oligotrophic nature of the isolate, which could probably grow on traces of organic contaminants in the components of BBMC medium, because no other energy source was added in the medium. No oxidation of As(III) was observed in un-inoculated controls (data not shown), indicating that As(III) oxidation was biologically mediated. The effect of vitamins and YE on the chemolithotrophic oxidation of As(III) by As3-1b is reported in Fig. 1b, c. The added concentration of YE, corresponding to 0.01 % (w/v), is a low concentration of organic carbon, probably mixotrophic conditions need higher concentration of organic substrate. Although the described strain did not show vitamins requirement, when vitamins were added in a defined formula to the culture medium, the added YE could represent a source of eventual different vitamins, or in different concentration able to stimulate the bacterial growth, but not the As(III) oxidation rate. Based on direct cell counts, the doubling time (t_d) was shortened in the presence of YE, changing from 8.1 (0 % YE) to 4.3 h (0.01 % YE). The enhancement of the specific As(III) oxidation rate in the presence of YE indicated that As3-1b gained energy from both the YE and As(III). The growth of As3-1b in chemolithotrophic and mixotrophic conditions was sustained in a successive re-fed of the culture with 3 mM of As(III), after complete transformation of the initial As(III) added to the cultures (Fig. 1). In the inoculated As3-1b culture broths, the pH passed from 8.0 to 6.0 in 72 h, whereas the pH did not change in the un-inoculated media and in cultures without arsenite.

Table 1 Phenotypic characteristics of *A. dichloromethanicus* As3-1b

Characteristic	As3-1b
Cell morphology	Rods
Motility	−
Arsenite oxidation	+
Catalase	+
Arginine dihydrolase	−
Beta-galactosidase	+
Cytochrome-oxidase	+
Gelatinase	+
Lysine decarboxylase	−
Ornithine decarboxylase	−
Tryptophane desaminase	−
Urease	+
Acetoin production	w
H_2S production	−
Indole production	−
NO_2 production	+
Esculin hydrolysis	+
Acid production from	
Glucose	+
Arabinose	+
Mannitol	+
Melibiose	+
Inositol	+
Rhamnose	+
Sorbitol	+
Sucrose	+
Amygdalin	+
Utilization of	
Glucose	+
Arabinose	+
Maltose	+
Mannitol	+
Mannose	+
N-acetyl-glucosamine	+
Citrate	+
Gluconate	+
Adipic acid	+
Capric acid	−
Malic acid	+
Phenylacetic acid	−

\+ positive, − negative, *w* weakly positive

Identification of Arsenite Oxidase and RuBisCO Genes

The presence of *aioA* arsenite oxidase, *cbbL*, and *cbbM* for RuBisCO Type-I and Type-II was evaluated in the DNA of the strain. A gene fragment of the expected size was amplified with primers for *aioA*. The deduced aminoacid sequence had 99 % homology to large subunit of arsenite oxidase-1 gene of *Ancylobacter* sp. strain OL1 (ABJ55852). The phylogenetic analysis conducted with deduced aminoacid sequences from *aioA* genes of different bacteria indicated that the sequence of strain As3-1b clustered together with those of other Alpha-proteobacteria, clearly

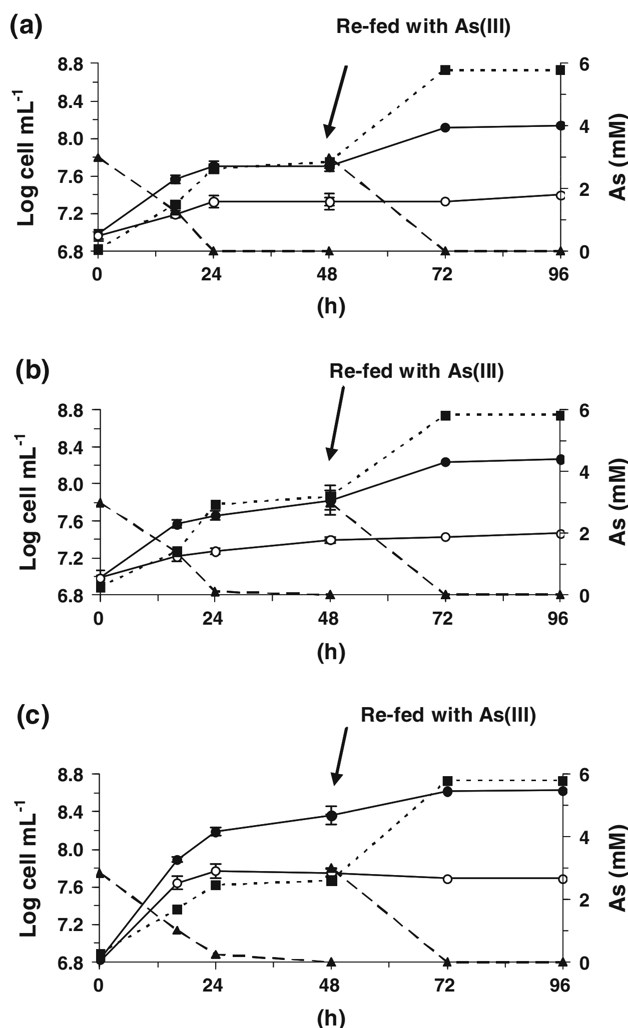


Fig. 1 As(III) oxidation and growth of *A. dichloromethanicus* As3-1b in: **a** BBMC, **b** BBMC lacking vitamin solution, and **c** BBMC + YE 0.01 %. After 48-h incubation time, the cultures were re-fed with extra 3 mM of arsenite. Each value is the media of three determinations. Growth with (*filled circle*) and without (*opened circle*) 3 mM As(III); As(III) (*filled triangle*); As(V) (*filled square*). *Bars*: ±SD where *bar* is absent, SD is within the *point*

distinguished from those of Beta- and Gamma-proteobacteria (Fig. 2).

As3-1b also carried a gene fragment homologous to RuBisCO Type-I *cbbL* gene, supporting the autotrophic nature of the strain As3-1b. In particular, the deduced aminoacid sequence of *cbbL* gene of strain As3-1b was highly homologous (99 % positive) to the large subunit of the RuBisCO Type-IC of *Starkeya novella* DSM 506 (YP_003692380.1) and to that of the uncultured bacterium clone L5 (EU450726; 87 % positive). The *cbbM* gene coding for Type-II RuBisCO was not detected in the strain As3-1b, although multiple attempts were carried out by using different PCR conditions.

Fig. 2 Phylogenetic relationships of arsenite oxidase 1 large subunit *aioA* gene (**a**) and ribulose-1,5-biphosphate carboxylase and oxygenase *cbbL* gene (**b**) (deduced amino acid sequence analysis) present in *A. dichloromethanicus* strain As3-1b. Phylogenetic trees were reconstructed from evolutionary distances by using the neighbor-joining method. *Bars* number of substitutions (%); the numbers at the nodes show bootstrap values greater than 70 % obtained from 1,000 resamplings. Strain discussed in this study is in *bold type* ▶

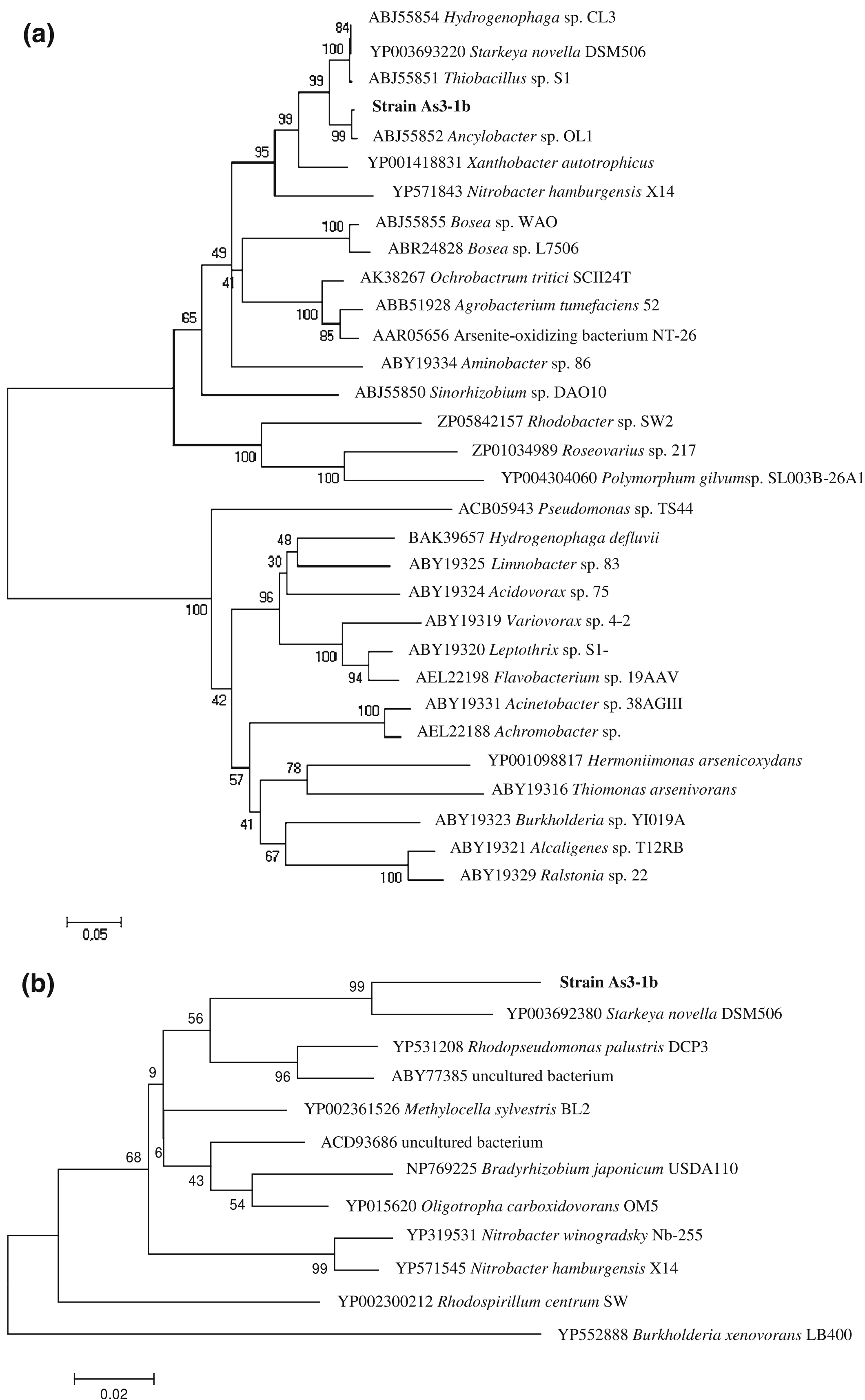
Discussion

Ancylobacter dichloromethanicus strain As3-1b can be considered as facultative chemolithotroph being able to grow either chemolithotrophically or chemoorganotrophically, similarly to the previously identified As(III)-oxidizers [10, 18, 20, 25]. The isolation of *A. dichloromethanicus* As3-1b from an agricultural As-polluted soil strengthens the finding that chemolithotrophic As(III) oxidizing bacteria are not restricted to extreme environments [8, 11, 12, 20], in accordance with Garcia-Dominguez et al. [10].

Strain As3-1b under lithotrophic condition has a t_d (8.1 h) comparable to that of NT-26 (7.6 h) [25], the most efficient As(III)-oxidizer until now, and a little bit faster than that of SDB1 (9.8 h) [17]. In the presence of YE (0.01 %), the t_d was reduced from 8.1 to 4.3 h. A such stimulatory effect of YE on the growth has been described also for NT-26 and SDB1 strains.

AioA detected in As3-1b strain was related to As(III) oxidases of previously reported chemolithotrophic As(III)-oxidizing Alpha-proteobacteria [10, 26]. Phylogeny of *aio*A deduced aminoacid sequence was inconsistent with the 16S rRNA phylogeny of the strain (see supporting material), in accordance with recent reports [13, 22] that support lateral gene transfer As(III) oxidase among bacterial populations. In addition to *aioA,* strain As3-1b harbored *arsC* and *arsB* genes [6], evidencing that in the strain multiple mechanisms were present to stand As toxicity either through As(V) reduction via ArsC and As(III) extrusion ArsB or through As(III) oxidation via AioA for gaining energy, as also described for *Herminiimonas arsenicoxydans* [19].

The autotrophic growth of *A. dichloromethanicus* As3-1b was dependent on As(III) oxidation, although the growth in the presence of other electron donors cannot be excluded. The autotrophic nature of the strain was confirmed by the presence of *cbbL* for RuBisCO Type-I. The sequence clustered together with RuBisCO Type-I of previously described chemolithotrophic Alpha-proteobacteria, but this is the first report identifying *cbbL* gene in *Ancylobacter* genus. *CbbM* gene was not retrieved in strain As3-1b, differently from *Ancylobacter* sp. strain OL-1 [10]. Phylogenetic models based on 16S ribosomal RNA not always reflect those based on RuBisCO in



(a)
ABJ55854 Hydrogenophaga sp. CL3
YP003693220 Starkeya novella DSM506
ABJ55851 Thiobacillus sp. S1
Strain As3-1b
ABJ55852 Ancylobacter sp. OL1
YP001418831 Xanthobacter autotrophicus
YP571843 Nitrobacter hamburgensis X14
ABJ55855 Bosea sp. WAO
ABR24828 Bosea sp. L7506
AK38267 Ochrobactrum tritici SCII24T
ABB51928 Agrobacterium tumefaciens 52
AAR05656 Arsenite-oxidizing bacterium NT-26
ABY19334 Aminobacter sp. 86
ABJ55850 Sinorhizobium sp. DAO10
ZP05842157 Rhodobacter sp. SW2
ZP01034989 Roseovarius sp. 217
YP004304060 Polymorphum gilvumsp. SL003B-26A1
ACB05943 Pseudomonas sp. TS44
BAK39657 Hydrogenophaga defluvii
ABY19325 Limnobacter sp. 83
ABY19324 Acidovorax sp. 75
ABY19319 Variovorax sp. 4-2
ABY19320 Leptothrix sp. S1-
AEL22198 Flavobacterium sp. 19AAV
ABY19331 Acinetobacter sp. 38AGIII
AEL22188 Achromobacter sp.
YP001098817 Hermoniimonas arsenicoxydans
ABY19316 Thiomonas arsenivorans
ABY19323 Burkholderia sp. YI019A
ABY19321 Alcaligenes sp. T12RB
ABY19329 Ralstonia sp. 22
0.05
(b)
Strain As3-1b
YP003692380 Starkeya novella DSM506
YP531208 Rhodopseudomonas palustris DCP3
ABY77385 uncultured bacterium
YP002361526 Methylocella sylvestris BL2
ACD93686 uncultured bacterium
NP769225 Bradyrhizobium japonicum USDA110
YP015620 Oligotropha carboxidovorans OM5
YP319531 Nitrobacter winogradsky Nb-255
YP571545 Nitrobacter hamburgensis X14
YP002300212 Rhodospirillum centrum SW
YP552888 Burkholderia xenovorans LB400
0.02

chemolithotrophic bacteria [31], although *cbbL* genes were reported as excellent markers for the phylogenetic analysis of autotrophic organisms [32].

In conclusion, *A. dichloromethanicus* strain As3-1b is an efficient As (III)-oxidizer able to oxidize 3-mM As(III) in 24 h under chemolithotrophic conditions. The ability of the strain to use As(III) and different C sources represents an advantage to survive in environments with changing conditions. Because of its ability to oxidize As(III) into the less toxic and more easily adsorbed As(V), strain As3-1b can be proposed for use in the first step of arsenic bioremediation processes.

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