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Adaptation mechanisms of *Alcanivorax* facilitating its predominance in marine environments

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Introduction: *Alcanivorax*, a typical alkane-degrading bacterium, has demonstrated the ability to utilize inorganic electron donor in some reports. However, a comprehensive analysis of its potentiality to utilize inorganic electron donor is still lacking.

Methods: In this study, genomic and phylogenetic analyzes were used to explore the potential oxidative capacity of inorganic compounds in *Alcanivorax*. And its functions were verified through physiological experiments.

Results: The sulfur oxidation-related genes *sqr* and *tsdA* are prevalent and have various evolutionary origins. Potential genes for CO oxidation were present in 39 strains, whereas genes associated with iron, hydrogen, and ammonia oxidation were either rare or absent. The physiological functions of Sqr and TsdA were confirmed in six representative strains under heterotrophic conditions. Adding thiosulfate enhanced *Alcanivorax* growth. However, *Alcanivorax* bacteria perform sulfide detoxification through Sqr rather than by gaining energy via sulfide oxidation Although no strain was confirmed to be chemoautotrophs, we discovered that the two clades, *A. xenomutans* and *A. profundimaris*, can grow under conditions with very low organic matter.

Discussion: The ability to utilize inorganic compounds as a supplementary energy source and adapt to carbon oligotrophic growth may contribute to the prevalence of *Alcanivorax* in marine ecosystems.

KEYWORDS

***Alcanivorax*, chemolithotrophic, carbon oligotrophic, sulfur oxidation, TsdA, Sqr**

Introduction

Marine microorganisms play a crucial role in marine ecosystems, playing a significant role in maintaining the balance of marine ecology and biogeochemical cycles. Generally, marine heterotrophs primarily utilize carbon from sinking organic particles (Engel et al., 2022), microbial remnants (Cerro-Gálvez et al., 2021; Katayama et al., 2024), dissolved organic matter (DOM) (Baltar et al., 2021; Quigley et al., 2019), and alkanes from the crust (Dong et al., 2022; Love et al., 2021). Interestingly, some studies have revealed that in areas where the concentration of organic matter is limited but the concentrations of reductive inorganic substances are high, certain groups of heterotrophic microorganisms, including *Alcanivorax*, are abundant (Dede et al., 2023; Chernikova et al., 2020; Kesava et al., 2020; Bhattacharya et al., 2020). When using chemoautotrophic culture media for enrichment, these heterotrophic groups are sometimes enriched (Dede et al., 2022; Wang et al., 2020; Wei et al., 2022; Hedrich et al., 2011). A new perspective suggests that *Alcanivorax* may be able to utilize these reductive inorganic substances in specific environments to achieve chemolithotrophic growth. Even fix inorganic carbon as the primary carbon source like chemoautotrophs (Wang et al., 2020). The main difference between chemolithotrophs and chemoautotrophs is where they get carbon. chemolithotrophs can get carbon from organic or inorganic sources and energy from inorganic sources. chemoautotrophs get both carbon and energy from inorganic sources.

Alcanivorax is a prominent genus that frequently emerges in studies of marine microorganisms (Wei et al., 2022; Wang et al., 2020). It is ubiquitous in oceanic environments, ranging from coastal areas to the deep sea, and even in hydrothermal vents (Yang et al., 2018; Dede et al., 2023; Chernikova et al., 2020; Wei et al., 2022; Wang et al., 2020). Its prevalence is notable, constituting approximately 0.1% of the microbial community in open ocean settings and exceeding 1% in coastal areas (Supplementary Figure 1, data from IMNGS (Lagkouvardos et al., 2016)).

The *Alcanivorax* genus is an important group within the class *Gammaproteobacteria*. The first strain of *Alcanivorax* was identified and named Yakimov in 1998, which initially gained significant attention due to its remarkable capacity to degrade alkanes (Yakimov et al., 1998). In past decades, an increasing number of *Alcanivorax* species have been isolated and identified, with a total of 29 species reported by 2024. However, according to the latest report, the phylotaxogenomic analysis showed that *Alcanivorax* species formed three clades. The original *Alcanivorax* genus has been further divided into *Alloalcanivorax*, *Isoalcanivorax*, and the narrow *Alcanivorax* (Rai et al., 2023).

Alcanivorax species are regarded as a group of traditionally heterotrophic microorganisms (Liu and Shao, 2005; Yang et al., 2018; Zhu et al., 2021). However, our recent report indicated that *Alcanivorax* bacteria can grow through iron oxidation (Wei et al., 2022). In hydrothermal environments, where alkanes and other organic compounds are not as abundant as reduced inorganic compounds (such as sulfide), their relative abundance can reach a significant level of up to 40% (Dede et al., 2023). These findings suggest that *Alcanivorax* may have special metabolic strategies for adapting to oligotrophic environments (Lv et al., 2024; Wang et al., 2020).

However, to date, there is no comprehensive investigation detailing the ability of *Alcanivorax* to oxidize inorganic reducing compounds. It is still unclear what types of inorganic energy it might use and whether it can fix inorganic carbon to achieve chemoautotrophic growth. In this study, we explored the aforementioned issues by analyzing the genomes of 167 *Alcanivorax* strains covering all 29 species and performing experimental validations on representative strains.

Materials and methods

Representative strains and cultural conditions

Six representative strains were selected base on phylogenetic tree and their genomes, which encode TsdA, Sqr and Sdo proteins, for the following experiments. These strains included *A. hongdengensis*^{T} MCCC1A01496, *Alcanivorax* sp. MCCC1A02275 (most similar to *A. borkumensis*), *Alcanivorax* sp. MCCC1A04522 (most similar to *A. modilis*), *A. venustensis* MCCC1A04970, *A. profundimaris*^{T} MCCC1A7714, and *A. xenomutans* MCCC1A05661. Strains *A. hongdengensis*^{T} MCCC1A01496, *Alcanivorax* sp. MCCC1A02275, *A. venustensis* MCCC1A04970, and *A. profundimaris*^{T} MCCC 1A7714 were isolated from upper seawater samples. *A. xenomutans* MCCC1A05661 was isolated from deep seawater sample, and *Alcanivorax* sp. MCCC1A04522 was specifically isolated from hydrothermal sediment sample. All strains were cultured on 2216E agar plates. Subsequently, the culture was thoroughly washed with phosphate buffered saline (PBS) three times before being inoculated into different media.

The composition of the heterotrophic sulfide (thiosulfate) oxidation media are as follows (L^{-1}): 2 g of acetate, 0.2 g of yeast, 25 g of NaCl, 0.5 g of KCl, 0.84 g of $NaNO_3$, 0.14 g of K_2HPO_4, 0.2 g of $NaHCO_3$, 1 mL of vitamins, 1 mL of trace elements (Balch et al., 1979), 1 mM $Na_2S{\cdot}9H_2O$ (10 mM $Na_2S_2O_4$). Furthermore, heterotrophic sulfide oxidation was tested under an anaerobic environment to avoid spontaneous sulfide oxidation in a short time. Another anaerobic heterotrophic growth experiment without sulfide was designed as a blank group to survey strains' growth ability under an anaerobic environment. One percent hexadecane was used to replace 2 g of acetate and 0.2 g of yeast was used for another heterotrophic thiosulfate oxidation experiment.

The composition of the chemoautotrophic thiosulfate oxidate media was as follows (L^{-1}): 25 g of NaCl, 0.5 g of KCl, 0.84 g of $NaNO_3$, 0.14 g of K_2HPO_4, 0.84 g of $NaHCO_3$, 1 mL of vitamins, 1 mL of trace elements and 20 mM $Na_2S_2O_4$.

Genomes annotation

Over the past two decades, 167 *Alcanivorax* genomes have been successfully sequenced by the Marine Culture Collection of China (MCCC). For translation and preliminary annotation analysis,

Prokka (v1.13) was used, while EggNOG-mapper (v2.0) was employed for a deeper exploration of metabolic pathways.

Key metabolic genes prediction

To identify functional genes responsible for reducing inorganic compound oxidation, the genes encoding proteins involved in sulfur oxidation (Sulfide: quinone reductase, Sqr; Flavocytochrome c sulfide dehydrogenase, FCSD; reverse dissimilatory sulfite reduction, Dsr; heterodisulide reductase-like, Hdr; sulfur oxygenase/reductase, Sor; sulfur dioxygenase, Sdo; thiosulfate oxidation complex, SoxABCDXYZ; adenylylsulfate reductase, Apr; sulfate adenylyltransferase, Sat; thiosulfate dehydrogenase, Tsd) (Hutt, 2017; Du et al., 2022; Zhang et al., 2020; Powell et al., 2017; Landry et al., 2021; Dahl et al., 2005), ammonia oxidation (ammonia monooxygenase, Amo; hydroxylamine oxidoreductase, Hao) (Kuypers et al., 2018; Stein, 2019), carbon monoxide oxidation (carbon monoxide dehydrogenase, CoxMSL) (Stein, 2019), and hydrogen oxidation (hydrogenase, Hya) (Abdellatif et al., 2017; Petersen et al., 2011) were identified by a local BLASTP protein similarity search with an amino acid similarity cutoff of 40%, coverage >80%, and an e-value cutoff of 1E-5. Additionally, the nitrogen-reducing genes were also surveyed to predict their ability to use nitrate as an electron acceptor via the same method. FeGenie was used to search for iron oxidation functional genes (Garber et al., 2020). The results obtained from both BLASTP and FeGenie were further checked against conserved domain search (CD-search) results (Lu et al., 2020).

Phylogenetic analysis

A genome-based phylogenetic tree encompassing 92 core genes was constructed by UBCG (v2) (bcg files were supported in Supplementary Material 1) (Na et al., 2018). The amino acid sequences of functional proteins, including Sqr and TsdA, were subjected to multiple sequence alignments with reference sequences obtained from the NCBI and UniProt databases (all sequences are listed in Supplementary Materials 2) via MAFFT (v7) (Katoh and Standley, 2013). Subsequently, these aligned sequences were employed in MEGA11, utilizing the JTT model and 1000 replicates, to construct individual neighbor-joining (NJ) phylogenetic trees for the respective functional genes (Tamura et al., 2021).

Verification of carbon fixation through $\delta^{13}C$ isotope analysis

In the ^{13}C isotopic tracer experiments for carbon fixation, the same medium used for chemoautotrophic thiosulfate utilization was used, and $NaHCO_3$ was replaced with $NaH^{13}CO_3$ as the sole source of the ^{13}C isotope. The cells were harvested from the heterotrophic medium (2216E) at 4500 rpm for 30 min, and then thoroughly washed three times with sterile chemoautotrophic medium to remove any residual organic matter. After completing the washing process, the concentration of the organisms was adjusted to OD_{600} = 0.6. Subsequently, the cells were inoculated into the chemoautotrophic medium at a concentration of 5% inoculum. The cells were then collected after 3 hours and 7 days of incubation. After centrifuging the cells at 8000 rpm for 20 min, the supernatant was discarded. To remove any residual $NaH^{13}CO_3$ adhering to the cells, they were washed by chemoautotrophic medium without isotopes and then centrifuged again for collection.

The ^{13}C isotopes of the cells were accurately determined with a stable isotope ratio mass spectrometer (IRMS) (measuring accuracy = ± 0.2; DeltaVAdvantage, Bremen, Germany).

Detection of total organic carbon

Total organic carbon (TOC) detection was conducted in a similar way to isotope experiments (but without the use of isotope-labeled media), and samples were individually collected on days 0, 7, and 21. The samples were acidified with hydrochloric acid (pH<2) before testing, and the acidification process was extended beyond 2 days to guarantee thorough elimination of inorganic carbon. TOC detection was then performed using a 900 Laboratory TOC Analyzer. To ensure the reliability of the results, three parallel samples were used for both the isotope test and the TOC measurement.

Detection of sulfur compounds

Sulfide was detected through the methylene blue method (Trüper and Schlegel, 1964), while thiosulfate, sulfite, and tetrathionate were detected through the spectrophotometric iodometric method, which relies on measuring the optical density at OD_{350}. When thiosulfate and sulfite are both present in a mixture, sulfite can be masked by formaldehyde. In the event of sufficient alkalinity, 2 mol of tetrathionate will yield 3 mol of thiosulfate and 2 mol of sulfite. Given that 2 μM of thiosulfate and 1 μM of sulfite both result in an identical decrease in OD_{350}, the sulfite and tetrathionate concentrations can be accurately calculated after the addition of formaldehyde and sufficient alkaline, respectively. The sulfate content in the cultures was determined by a turbidimetric method (Kolmert et al., 2000).

Results and discussion

Basic genomes and phylogenomic analysis

A total of 167 genomes were counted, with sizes ranging from 3.05 to 5.09 Mb. Among them, the 108 genomes included 17 species, mainly *A. borkumensis*, *A. marinus*, *A. profundimaris*, *A. venustensis*, *A. xenomutans*and, and 12 other species. An additional 59 isolates have not yet been definitively classified into a definite species. These isolates were collected from diverse environments, including deep-sea water, deep-sea sediments,

hydrothermal sediments, and upper seawater (Figure 1, with further details provided in Supplementary Table 1). A phylogenomic tree encompassing 92 core genes was constructed by UBCG (v2). Based on the phylogenetic tree results and the original strain names, numerous clades were delineated (Figure 1).

Identification of potential genes involved in chemolithotrophic growth

Using local BLASTP, Sqr was identified in 110 of 167 genomes, while Sdo was detected in 153 genomes. Sqr is predominant present in *A. hongdengesis*, *A. nanhaiticus*, *A. sediminis*, *A. jadensis*, *A. borkumensis*, *A. venustensis*, *A. xenomutans*, *A. balearicus*, and *A. profundimaris*, but is mainly absent in *A. limicola*, *A. pacificus*, and *A.profundi*. Sdo was prevalent in most genomes except for those of *A. limicola* and *A. pacificus*. Notably, all strains harboring the *sqr* gene also exhibited the presence of the *sdo* gene upstream, indicating a potential strategy for efficient sulfide oxidation in environments with high sulfide concentrations (Ran et al., 2022; Xia et al., 2017; Wang et al., 2014; Wu et al., 2017; Cuevasanta et al., 2017). TsdA was identified in a total of 70 genomes, including those of *A. xenomutans*, *A. profundimaris*, *A. hongdengesis*, *A. gelatinphageus*, *A. marinus*, and *A. mibilis*. This finding aligns with the results obtained from Prokka analysis.

Despite attempts at local BLASTP and a 40% identity threshold, certain genes crucial for sulfur oxidation, such as the *sox* complex, *apr*, and *dsr*AB, remained undetected. Similarly, genes associated with nitrogen and hydrogen oxidation were also elusive, in line with the results obtained from direct annotation using Prokka. However, a potential *cox*LMS operon was identified in species such as *A. profundimaris, A. gelatinphageus*, *A. marinus*, and several unnamed strains closely related to *A. marinus*, with a total of 39 sequences encoding carbon monoxide dehydrogenase (further details are

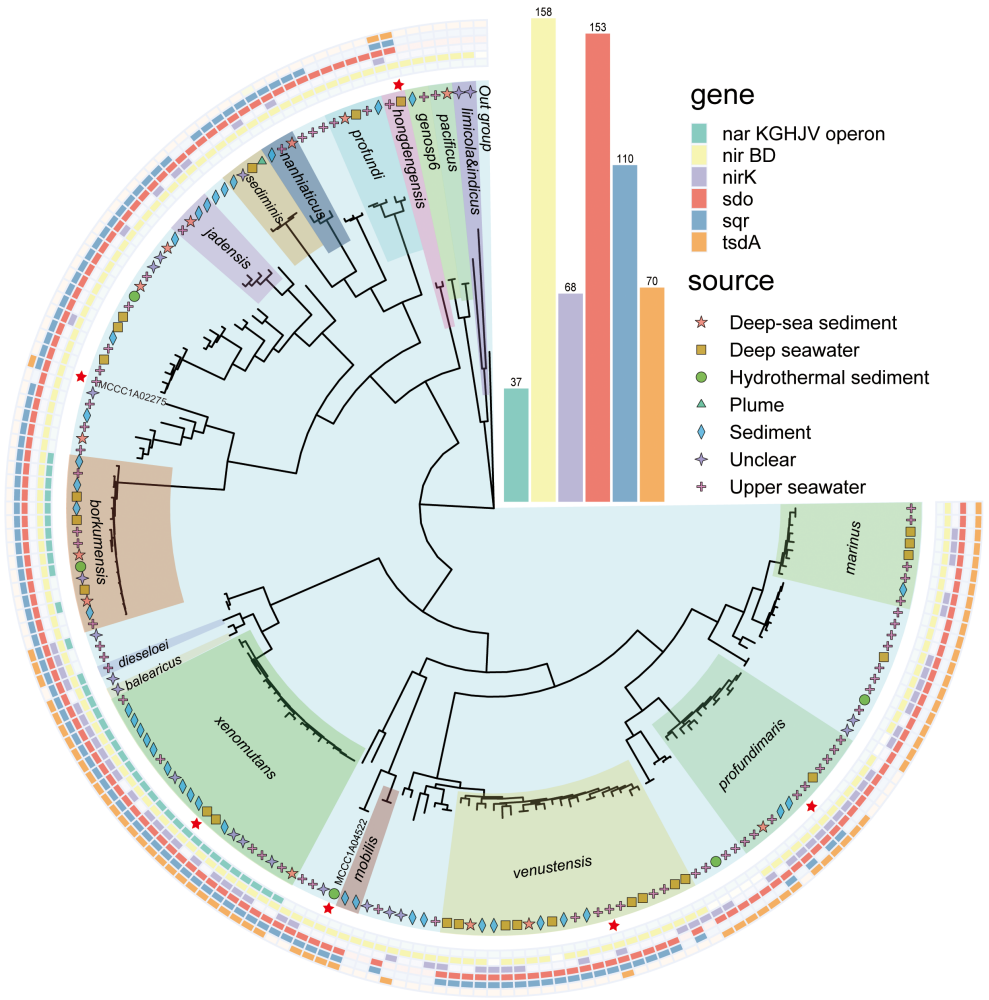

FIGURE 1
Phylogenetic tree of the genus *Alcanivorax* constructed using UBCG(v2). The different colors inside represent different species of *Alcanivorax*, which were divided into 17 clades, and unnamed strains not showing names in this tree without a color block. The inner symbol indicates the strain source. The red stars are representative strains used for the following experiments. The outside ranges with different colors show the results of functional genes identified in their genomes. The inside out indicates the nar operon, *nir*BD, *nir*K, *sdo*, *sqr*, and *tsd*A.

provided in Supplementary Material 3). Notably, despite BLASTP identification falling below the 40% identity threshold, these sequences exhibit functional domains similar to those of CoxLMS. Moreover, attempts to identify the iron oxidase gene using Fegenie revealed the presence of Cyc1 in two strains, *Alcanivorax* sp. MCCC1A05192 and MCCC1A04750. Genes commonly associated with iron oxidation, including Cyc2, the Fox operon, Sulfocyanin, PioABC, and MotAB, as reviewed by Garber, were conspicuously absent in the genomes (Garber et al., 2020).

Over one-third of the strains showed the presence of the *tsd*A gene, while more than two-thirds contained the *sqr* gene, which is responsible for sulfide oxidation. Additionally, *sdo* genes were nearly ubiquitous across all strains. The analysis of iron oxidation genes in individual bacterial species aligned with previous reports (Wei et al., 2022), indicating the potential for iron oxidation among these strains. These findings suggest that *Alcanivorax* bacteria may have a diverse range of available inorganic energy sources. Traditionally, *Alcanivorax* strains have been regarded as typical heterotrophic bacteria capable of utilizing various carbon sources, including some recalcitrant compounds. However, a recent breakthrough in 2022 revealed that a strain of *Alcanivorax* exhibits remarkable iron oxidation capabilities and potential for carbon fixation (Wei et al., 2022), thereby challenging the long-held perception of *Alcanivorax* as purely heterotrophs. This discovery suggested that they may belong to a group of chemolithotrophs.

Identification of nitrogen-reducing genes

There is no doubt that *Alcanivorax* strains can use oxygen as an electron acceptor (Liu and Shao, 2005; Zhu et al., 2021), but it is still unclear whether they can use nitrate as an electron acceptor under microaerobic or anaerobic conditions. Therefore, the nitrogen-reducing genes of *Alcanivorax* were also surveyed.

The ability to perform complete assimilative nitrate reduction is a feature found across all *Alcanivorax* genomes. However, the capacity for dissimilatory nitrate reduction is restricted to certain clades. Notably, the *nar*KGHJV operon, which is essential for converting nitrate to nitrite (Kuypers et al., 2018), is unique to the *A. barkumensis* and *A. xenomutans* clades. In contrast, the NirBD system, which is responsible for the conversion of nitrite to ammonium, is nearly universally present across nearly all genomes. Furthermore, within the denitrification pathway, NirK, which catalyzes nitrite to nitric oxide, was identified in 68 genomes, predominantly within the *A. xenomutans*, *A. venustensis*, *A. profundimaris*, and *A. mariuis* clades.

Nitrate reduction genes are widespread among *Alcanivorax* species, indicating their potential to use nitrate as an electron acceptor, thereby facilitating electron transfer within respiratory chains (Kuypers et al., 2018). This capability is particularly relevant in environments where thiosulfate or sulfide accumulates under microaerobic or anaerobic conditions. *Alcanivorax*, a commonly encountered and abundant bacterial group in marine environments (Dede et al., 2023), possesses a high proportion of genes related to sulfur oxidation and nitrate reduction, suggesting that it plays a significant role in the inorganic nitrogen and sulfur cycles in marine environments.

Potential carbon fixation pathway analysis

To date, a total of nine distinct pathways for microbial fixation of inorganic carbon have been reported, including six classical natural pathways and three alternate pathways Mall et al., 2018; Sánchez-Andrea et al., 2020; Steffens et al., 2021; Correa et al., 2023). However, despite thorough investigations, neither the entire suite of microbial inorganic carbon fixation pathways nor their crucial genes have been comprehensively identified in *Alcanivorax* (Supplementary Material 4). Nevertheless, the reverse Tricarboxylic Acid cycle (rTCA) and Calvin-Benson-Bassham cycle (CBB) pathways share relatively high integrity in *Alcanivorax*. Unfortunately, the key genes of the rTCA and CBB pathways were all absent.

In the rTCA pathway, most of the genes can be found in genomes. Although a few genes are missing in some genomes, they are not absent in special species. For instance, Citryl-CoA lyase (*ccl*) is present in the genomes of *A. xenomuant* and *A. profundimaris*. However, the key genes ATP-citrate lyase (*acl*) and citryl-CoA synthetase (*ccs*), which are considered essential as key genes, are absent from all the genomes.

In the CBB pathway, the crucial functional gene ribulose-bisphosphate carboxylase is conspicuously absent from the genomes investigated. Only a few genes encoding fructose-bisphosphate aldolase (Fba) and phosphoglycerate kinase (Pgk) were identified. Based on the finding that most of the genes in the CBB pathway are absent, it seems improbable that carbon fixation would occur through the CBB pathway in these organisms.

Phylogenetic analysis of Sqr and TsdA

The phylogenetic tree of Sqr of *Alcanivorax* exhibited similarity to the reported classification (Marcia et al., 2010), with a clear division into FCSDs and six types of Sqr. All Sqr homologs of *Alcanivorax* belonged to type II, except for a single copy of *Alcanivorax* sp. MCCC1A05192, which was categorized as type I (Figure 2). They were categorized into three distinct clusters. Except for the single copy of *Alcanivorax* sp. MCCC1A05192, all others exhibited proximity to the neighboring branches of *Pseudomonas aeruginosa* and other related genera of *Gammaproteobacteria*. The Sqr close to *Alcanivorax* predominantly was origin from heterotrophic bacteria and some eukaryotes, whereas the reference sequences located in the more distant cluster within type II Sqr are mostly derived from chemoautotrophs, such as *Sulfurimonas*. In addition, the type I Sqr of *Alcanivorax* sp. MCCC1A05192 exhibits the highest similarity to the Sqr of *Acidithiobacillus ferrooxidans*, a strain that is evolutionarily remote from *Alcanivorax*.

Phylogenetic analyses revealed that the Sqr branches of *Alcanivorax* clustered, characterized by a high degree of Sqr similarity within the genus, indicating minimal variability and pronounced homology with Sqr originating from neighboring species.

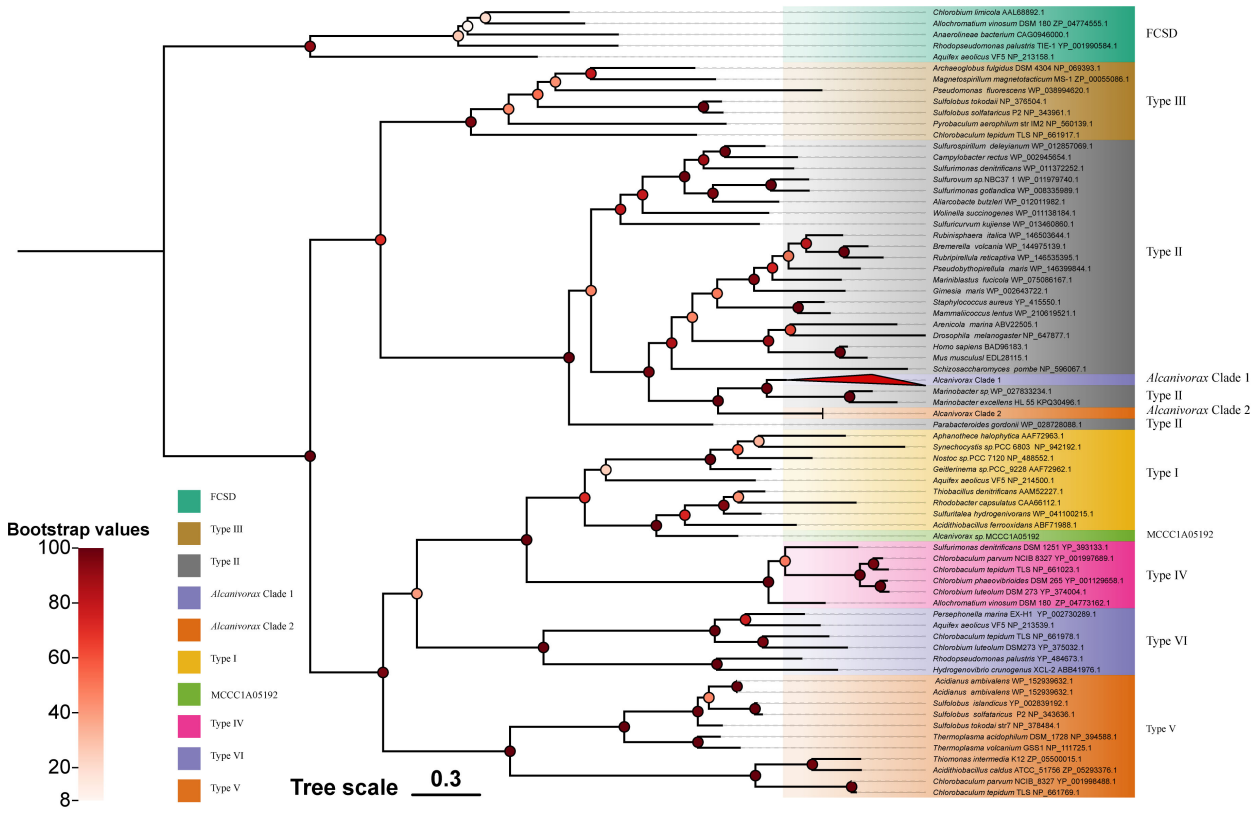

FIGURE 2
Phylogenetic tree of *Alcanivorax* and reference Sqr sequences. The different square blocks represent the FCSD, six-type Sqr, and *Alcnivorax* clades or strains. The red triangle block (*Alcanivorax* clade I) contains most of the Sqr sequence branches of *Alcanivorax*, counting 107.

Among the type II Sqr, the Sqr from chemoautotrophs and heterotrophic bacteria are clearly divided into two distinct branches. This separation suggests that type II Sqr enzymes may have evolved from two distinct common ancestors. Additionally, the Sqr of *Alcanivorax* originated from heterotrophic ancestors. Type II Sqr primarily perform detoxification functions in organisms, as has been demonstrated in mitochondria and diverse bacterial species (Marcia et al., 2009; Powell et al., 2017; Szabo, 2018). Although type II Sqr are also present in chemoautotrophic sulfide bacteria, their genomes are also characterized by other types of Sqr, such as type IV-V Sqr, which have been confirmed to support chemoautotrophic growth when sulfide serves as the sole electron donor (Han and Perner, 2015). Hence, it is difficult to determine whether type II Sqr provides sufficient energy to support the growth of *Alcanivorax*, which needs to be verified by wet experiments as follows.

The phylogenetic tree of TsdA shows that the TsdA genes of *Alcanivorax* are grouped into two distinct clades (Figure 3). Clade I, comprising 35 members, includes *A. marinus*, *A. gelattinphagus*, *A. profundimaris*, *A. hongdengsis*, and several unnamed strains. Clade II, with 36 members, encompasses *A. xenomutans*, *A. mobilis*, *A. balearicus*, and a subset of *A. profundimaris*. Notably, the most recent branches within clade I TsdA exclusively comprise chemoautotrophs that have been documented to be phototrophs (Kurth et al., 2016). However, it has been reported that the neighboring branches of the two clades are predominantly composed of TsdA originating from common heterotrophic sulfur-oxidizing taxa, which are distributed in distinct clusters compared to the TsdA of chemoautotrophs. After a thorough analysis of the protein cluster arrangements of TsdA in both classes, it became evident that the TsdA of these two classes exhibited significant differences in their genomic organization. Specifically, TsdB was observed upstream of TsdA in clade II, whereas its counterpart was conspicuously absent in clade I. Even though TsdA does not rely on TsdB or cytochrome c4 as an electron acceptor, some microorganisms can still function independently (Denkmann et al., 2012). The absence of TsdB in clade I attracted our attention, given that the TsdA of clade I are significantly longer (by 220 amino acid residues, Supplementary Materials 5) than the TsdA of clade II and the reported TsdA. Furthermore, the three neighboring branches harbor taxa in which TsdBA fusions have been documented (Kurth et al., 2016). A subsequent CD-search analysis of the conserved structures within certain sequences revealed that the initial approximately 200 amino acid residues of clade I belong to the Cyt553 superfamily, the same protein superfamily as TsdB (Lu et al., 2020). In conclusion, type I TsdA fused with TsdB, analogous to the phototrophic groups *Thiocystis violascens*, *Thioflavicoccus mobilis*, and *Marichromatium purpuratum* (Kurth et al., 2016; Yu, 2022). This marks the first discovery of two TsdA types within a single genus and even within a single strain (*A. profundimaris*).

Despite the seemingly diverse origins of the two types of TsdA found in *Alcanivorax*, they exhibit remarkable structural similarity

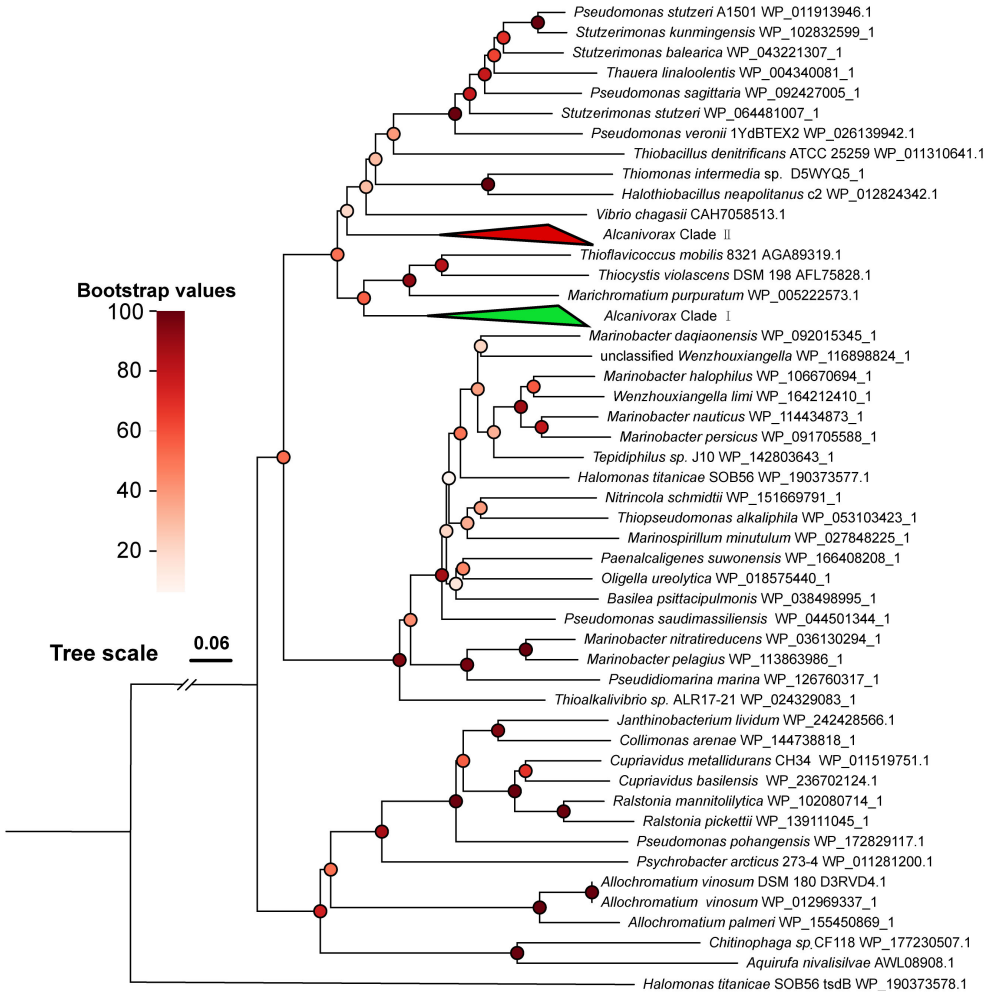

FIGURE 3
Phylogenetic tree of *Alcanivorax* and reference TsdA sequences. The green triangle block (*Alcanivorax* clade I) contains 35 branches of *Alcanivorax*, including TsdA of *A. marinus*, *A. gelattinphagus*, *A. profundimaris*, and *A. hongdengsis*. The red triangle block (*Alcanivorax* Clade II) contains 36 branches of *Alcanivorax*, including Tsd(B)A of *A. xenomutans*, *A. mobilis*, *A. balearicus*, and a subset of *A. profundimaris*.

and perform analogous functions with minor variations in their enzymatic characteristics (Brito et al., 2015; Kurth et al., 2016; Jenner et al., 2019). When Tsd(B)A oxidates thiosulfate, the two electrons that are produced are typically presumed to be relayed to TsdB or HiPIP proteins, subsequently conveyed to cytochrome cbb3, and ultimately integrated into the respiratory chain. The electron transfer process of the fusion variant of Tsd(B)A not only involves direct transport to cytochrome cbb3 but also has the potential to integrate into the electron chain of the photosynthetic reaction (Kurth et al., 2016). Despite these diligent searches, no photosynthetic pigment genes have been discovered in *Alcanivorax*, thus eliminating the prospect of its involvement in photosynthetic reactions for electron transfer. As a result, the electron transfer mechanism for both types of Tsd(B)A in *Alcanivorax* is expected to be analogous, ultimately culminating in their integration into the respiratory chain to generate reducing power, which then provides energy for growth. In *M. purpuratum*, studies have demonstrated that the fused type of Tsd(B)A exhibits a heightened affinity for substrates (Kurth et al., 2016), suggesting that the presence of various Tsd(B)A types may be essential for accommodating different thiosulfate concentrations.

Heterotrophic sulfur oxidation assays by representative strains

Six representative strains of *Alcanivarax* were selected to confirm the physiologic functions of Sqr and TsdA in media including artificial seawater, sulfide (or thiosulfate), and organic matter (acetic and yeast, or hexadecane). The sulfide oxidation experiment showed that all six strains were capable of promoting sulfide oxidation in an aerobic environment. Moreover, the efficiency of sulfide oxidation was comparable among all six strains, as sulfide was reduced from 1 mM to approximately 0.3 mM by each strain, while the noninoculated group still retained 0.5 mM sulfide within 60 minutes. After 180 minutes, 1 mM sulfide was completely consumed by the strains, whereas the noninoculated group still contained 0.1 mM residual sulfide (Figure 4).

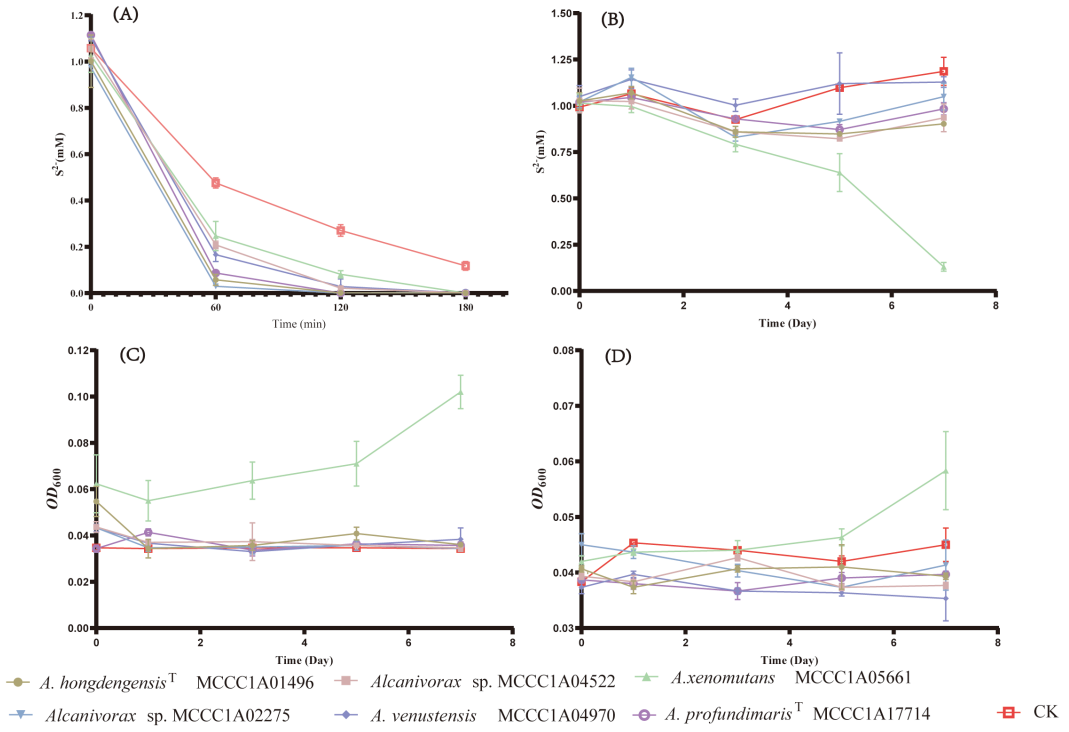

FIGURE 4
The growth and sulfide oxidation of representative strains under aerobic and anaerobic conditions. **(A)** Changes in the concentration of aerobic heterotrophic sulfide, **(B)** Changes in the concentration of anaerobic heterotrophic sulfide, **(C)** Changes in the anaerobic heterotrophic OD_{600} without sulfide, **(D)** Changes in the anaerobic heterotrophic OD_{600} with 1mM sulfide.

Under heterotrophic anaerobic conditions, *A. xenomutans* MCCC1A05661 was the sole strain exhibiting growth and sulfide oxidation capabilities. For seven days, the sulfide concentration markedly decreased by 0.88 mM, accompanied by a corresponding increase in biomass, as indicated by the increase in the OD_{600}, which increased from 0.04 to 0.06. Conversely, the OD_{600} of the control group, which did not receive any sulfide, exhibited a more significant increase, reaching 0.1 of the OD_{600}. After seven days, the sulfide concentration and OD_{600} values of the other five strains, as well as those of the blank control group, remained almost unchanged. Based on the quantification results of *A. xenomutans* MCCC1A05661, we observed a substantial inhibitory impact on the growth of *A. xenomutans* MCCC1A05661 (Supplementary Figure 2). This suggests that sulfide exerts a specific toxic effect during the growth phase of *Alcanivorax*. While sulfide may release a certain quantity of energy through the oxidation process by Sqr, its harmful impact on cellular toxicity significantly outweighs its marginal positive influence on cell growth.

Therefore, the primary role of Sqr in *Alcanivorax* is to facilitate the detoxification of sulfide rather than contributing significantly to the growth of sulfur-oxidizing bacteria such as *Beggiatoa*, which are capable of utilizing sulfide as an energy source (Berg et al., 2014). Furthermore, the inability of the remaining five strains to grow is likely attributed to the absence of the Nar operon, which is essential for effectively utilizing nitrate as an electron acceptor (Stein, 2019). Given the experimental conditions outlined in this study, it is highly probable that these five strains lack suitable electron acceptors.

The oxidative ability of thiosulfate was observed under both heterotrophic and chemoautotrophic conditions. Among the heterotrophic cultures with sodium acetate and yeast, *A. xenomutans* MCCC1A05661, *A. venstensis* MCCC1A04970, *Alcanivorax* sp. MCCC1A02275 and *A. profundimaris*^{T} MCCC1A7714 were able to oxidize thiosulfate, whereas *A. hongdengensis*^{T} MCCC1A01496 and *Alcanivorax* sp. MCCC1A04522 exhibited no such activity. Notably, *A. xenomutans* MCCC1A05661 showed the lowest ability to oxidize thiosulfate under heterotrophic (acetate and yeast) conditions. Within 48 hours, *A. xenomutans* MCCC1A05661 consumed 1 mM thiosulfate and produced 0.5 mM tetrathionate. In contrast, the other species consumed 5 to 8 mM thiosulfate and generated 2 to 4 mM tetrathionate within the same period (Figures 5A–C). However, when hexadecane served as the sole source of carbon and energy, all strains exhibited the ability to oxidize thiosulfate. They consumed 5 mM thiosulfate and produced 2 to 3 mM tetrathionate within 2 days (Figures 5D–F). Notably, under heterotrophic conditions, the addition of thiosulfate significantly enhanced the growth of the bacteria, regardless of whether sodium acetate with yeast or hexadecane was the primary carbon source (Supplementary Figures 3, 4).

In heterotrophic sulfur oxidation experiments, the introduction of inorganic energy sources has been shown to stimulate bacterial growth. This finding implies that *Alcanivorax* may belong to a neglected class of chemolithotrophs rather than heterotrophs. The utilization of sulfur oxidation as an auxiliary energy source could explain the prevalent abundance of *Alcanivorax* in sulfur-rich

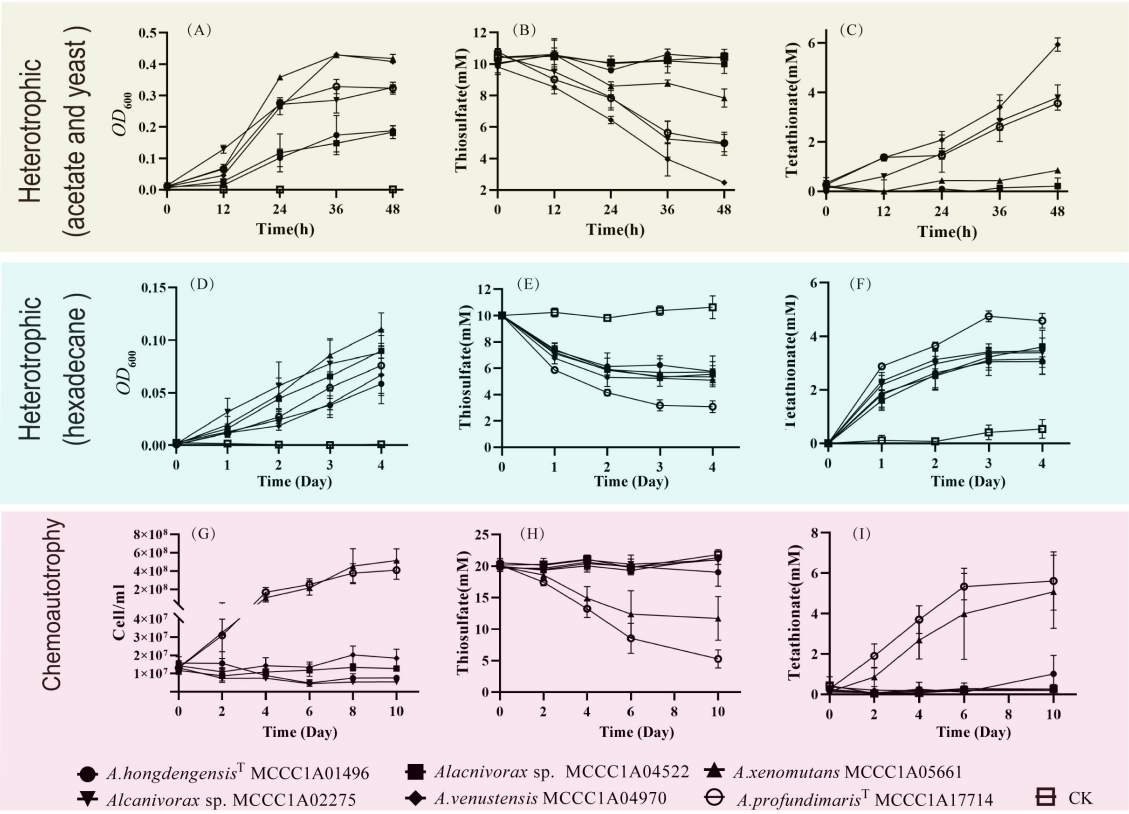

FIGURE 5
The growth and thiosulfate oxidation of *Alcanivorax* under different conditions. **(A–C)** Changes in OD_{600}, thiosulfate, and tetrathionate in acetate and yeast heterotrophic sulfur-oxidizing media; **(D–F)** Changes in OD_{600}, thiosulfate, and tetrathionate under hexadecane heterotrophic conditions; **(G–I)** Changes in cell concentration, thiosulfate and tetrathionate in chemoautotrophic media.

environments, such as hydrothermal fluids (Salazar et al., 2016; Wang et al., 2020; Dede et al., 2023);. Although it has been postulated that TsdA-mediated oxidation of thiosulfate results in an increase in pH, thereby indirectly fostering bacterial growth, we cannot discount this potential effect. However, it is noteworthy that the electrons generated during this oxidation process ultimately feed into the respiratory chain, supplying a considerable amount of energy. The impact of this energy derived from thiosulfate oxidation, along with changes in culture conditions such as pH, varies among different bacterial strains (Dlu, 2003; Hutt, 2017). It is now generally accepted that the energy derived from TsdA-mediated thiosulfate oxidation, in the absence of *soxB*, is insufficient to sustain bacterial growth and is considered a supplementary energy source (Denkmann et al., 2012). This finding aligns with the results observed under the culture conditions established in the current study. However, following the silencing of the *soxB* gene in *Paracoccus thiocyanatus*, which naturally harbors both the TsdA and SoxB oxidation systems, the strain showed typical features of TsdA-mediated thiosulfate oxidation. Notably, the cell density initially increased from

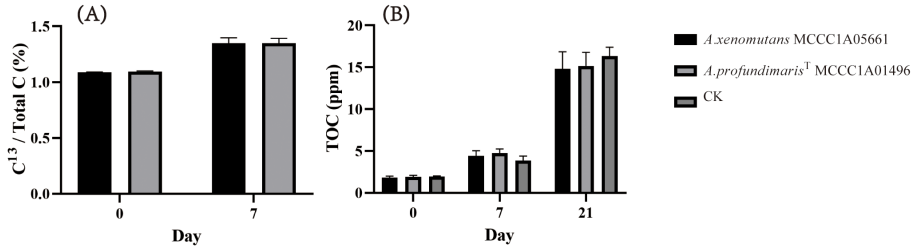

FIGURE 6
Carbon fixation ability of *A xenomuants* MCCC1A05661 and *A profundmaris*^{T} MCCC1A7714. **(A)** $\delta^{13}C$ assimilation, **(B)** TOC changes under chemoautotrophic conditions.

approximately 5×10^6 cells/mL to 5×10^7 cells/mL but subsequently decreased to 1×10^7 cells/mL upon complete consumption of thiosulfate (Rameez et al., 2020). Hence, despite the previous assumption that the energy produced by TsdA oxidation of thiosulfate solely functions as a supplementary energy source, the potential for this process to support bacterial growth remains a topic of discussion (Denkmann et al., 2012; Hutt, 2017).

Oligotrophic growth and inorganic carbon fixation in *Alcanivorax*

By exploring the potential for *Alcanivorax* to utilize thiosulfate for chemoautotrophic growth, we discovered that the *A. xenomuants* MCCC1A05661 and *A. profundmaris*^{T} MCCC1A7714 displayed thiosulfate oxidation ability, but others did not exhibit significant alterations in thiosulfate or tetrathionate levels. *A. xenomuants* MCCC1A05661 consumed approximately 8 mM thiosulfate and converted it to 4 mM tetrathionate, and *A. profundmaris*^{T} MCCC1A7714 exhibited a similar pattern, utilizing 15 mM thiosulfate to produce 7 mM tetrathionate in 10 days (Figures 5H, I). The cells underwent a remarkable increase in cell density during 10 days of chemoautotrophic culture, growing from approximately 1.0×10^7 cells/L to approximately 5.0×10^8 cells/L. However, others did not show any notable changes in cell numbers (Figure 5G). It seems that the *A. xenomuants* MCCC1A05661 and *A. profundmaris*^{T} MCCC1A7714 utilize thiosulfate for chemoautotrophic growth. However, the results of ^{13}C isotope analysis and total organic carbon (TOC) measurements fail to substantiate the possibility of chemoautotrophic growth via thiosulfate oxidation. Despite the isotope experiments indicating that a fraction of inorganic carbon entered the cytosol, the assimilation rate increased only marginally from 1.1% to 1.3% (Figure 6A). This percentage is even lower than the assimilation rate of inorganic carbon by *Bacillus subtilis* under heterotrophic conditions, which is 5% (Braun et al., 2021). Hence, the assimilation of inorganic carbon likely occurs through a replenishment pathway, which usually occurs in heterotrophs. The TOC results showed that after 21 days of culture, the organic matter concentration increased from 2 ppm to 15 ppm in the CK group (Figure 6B), suggesting that these two strains were oligotrophs. These observations suggest that the carbon source sustaining the growth of *A. xenomuants* MCCC1A05661 and *A. profundmaris*^{T} MCCC1A7714 under the specified chemoautotrophic conditions is not primarily inorganic carbon, most likely due to air pollution.

Although the *A. xenomuants* MCCC1A05661 and *A. profundmaris*^{T} MCCC1A7714 cannot efficiently fix inorganic carbon, they exhibit remarkable resilience, demonstrating their capacity to thrive in extremely oligotrophic conditions, even at concentrations of organic matter as low as 5 ppm or below. Notably, similar results were obtained for other strains of *A. xenomuants* and *A. profundmaris* (data not show). However, the other strains lacked similar adaptability. This suggests that the capacity to proliferate under extreme oligotrophic conditions may not be a universal trait among *Alcanivorax* species; rather, it may indicate species or substrate specificity. Consequently, the presence of distinct trace organics in various regions may significantly contribute to the prevalence of specific *Alcanivorax* types in the pelagic environment.

Summary and conclusions

This study investigated the metabolic characteristics of *Alcanivorax* species in marine environments, particularly their ability to utilize inorganic compounds. The genes associated with sulfur oxidation, *sqr* and *tsd*A, were prevalent, and their distribution was closely tied to species evolution rather than habitat.

Experimental validations confirmed that the addition of thiosulfate can enhance *Alcanivorax* growth, suggesting its potential as a supplementary energy source. However, *Alcanivorax* bacteria primarily use Sqr for sulfide detoxification rather than for energy acquisition through sulfide oxidation. Although no strains were confirmed as chemoautotrophs, certain lineages, *A. xenomutans* and *A. profundimaris*, can grow under conditions with very low organic matter. The potential for chemolithotrophy and carbon oligotrophy likely drives the widespread distribution of *Alcanivorax*. Further research is needed to fully understand the mechanisms underlying this adaptation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

ZC: Writing – review & editing, Writing – original draft, Software, Methodology. SX: Writing – review & editing, Data curation. YL: Writing – original draft. QL: Writing – review & editing. CD: Writing – review & editing. JL: Writing – review & editing. GL: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition. ZS: Writing – review & editing, Writing – original draft, Methodology, Data curation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1491690/full#supplementary-material

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