Comparative insights into genome signatures of ferric iron oxide- and anode-stimulated Desulfuromonas spp. strains

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Abstract

Background: Halotolerant Fe (III) oxide reducers affiliated in the family Desulfuromonadaceae are ubiquitous and drive the carbon, nitrogen, sulfur and metal cycles in marine subsurface sediment. Due to their possible application in bioremediation and bioelectrochemical engineering, some of phylogenetically close Desulfuromonas spp. strains have been isolated through enrichment with crystalline Fe (III) oxide and anode. The strains isolated using electron acceptors with distinct redox potentials may have different abilities, for instance, of extracellular electron transport, surface recognition and colonization. The objective of this study was to identify the different genomic signatures between the crystalline Fe (III) oxide-stimulated strain AOP6 and the anode-stimulated strains WTL and DDH964 by comparative genome analysis.

Results: The AOP6 genome possessed the flagellar biosynthesis gene cluster, as well as diverse and abundant genes involved in chemotaxis sensory systems and c-type cytochromes capable of reduction of electron acceptors with low redox potentials. The WTL and DDH964 genomes lacked the flagellar biosynthesis cluster and exhibited a massive expansion of transposable gene elements that might mediate genome rearrangement, while they were deficient in some of the chemotaxis and cytochrome genes and included the genes for oxygen resistance.

Conclusions: Our results revealed the genomic signatures distinctive for the ferric iron oxide- and anode-stimulated Desulfuromonas spp. strains. These findings highlighted the different metabolic abilities, such as extracellular electron transfer and environmental stress resistance, of these phylogenetically close bacterial strains, casting light on genome evolution of the subsurface Fe (III) oxide reducers.

Keywords: Comparative genomics, Chemotaxis, Extracellular electron transport, c-type cytochrome, Flagellum, Cytochrome c oxidase, Transposable element, Desulfuromonas
specific c-type cytochromes can establish a conductive pathway capable of crossing the inner membrane, periplasm and outer membrane that allows the Geobacter species to directly transport electrons from the quinone/quinol pool to metal oxides [12, 17]. Additionally, these bacteria produce the electrically conductive protein nanowires, i.e., the e-pili assembled from the PilA pilin monomer, to achieve long-range extracellular electron transport to metal oxide particles [16]. Compared to metal oxides serving as electron acceptor, constant voltage electrodes represent the unlimited electron-accepting potentials allowing microorganisms in contact with the inorganic surface to support the growth of the other distant ones, if these microorganisms can create a conductive network to relay electrons to the electrode [18]. A number of studies showed that some of metal-reducing bacteria utilized the distinctive electron transfer proteins to access the electrodes. Chan and the colleagues constructed the genome-wide transposon-insertion sequencing libraries of G. sulfurreducens and found a new putative porin-cytochrome conduit complex (i.e., extABCD) crucial for growth with electrodes but not for Fe (III) oxide reduction [19]. Zacharoff et al. reported that deletion of the pgcA gene, encoding an extracellular triheme c-type cytochrome, in the G. sulfurreducens genome generated the mutants unable to transfer electrons to the Fe (III) and Mn (IV) oxides but able to maintain the ability of electrode reduction [20]. These studies suggest that functional diversification of c-type cytochromes from G. sulfurreducens was responsible for electron transfer to a variety of extracellular electron acceptors, particularly the metal oxides and electrodes.

Cell mobility and extracellular structures (e.g., type-IV pili) are critical for the Geobacter physiology, when Geobacter spp. are grown with insoluble Fe (III) oxides [21]. The related metabolic processes could be potentially regulated by chemotaxis-like pathways. Various chemotaxis systems and chemo-receptors (e.g., methyl-accepting chemotaxis proteins [MCPs]) have been well characterized in the Geobacter genomes and probably play distinct roles in different cellular processes [22]. For example, it is suggested that the E. coli-like and Frr-like clusters participate in the flagellum-based and social motilities, respectively, while the Dif-like and Deltaproteobacteria-specific clusters regulate the synthesis of extracellular matrix materials, such as exopolysaccharides [22]. Moreover, the G. sulfurreducens mutants lacking components of a methyl-accepting chemotaxis sensing network (i.e., extABCD) were defective in colonization on electrodes but grew normally with Fe (III) oxides, suggesting the distinct recognition and colonization mechanisms between the electrode and Fe (III) oxide reductions [19]. However, such detailed information on the other dissimilatory metal-reducing bacteria is still limited.

Apart from the Geobacteraceae bacteria inhabiting in soils and freshwater ecosystems, the Desulfuromonadaeae members, including the genera Desulfuromonas, Pelobacter, Desulfuromusa, Malomonas and Geoalkalibacter, are mainly isolated from marine sediments [23–26], tidal flat [27], petroleum reservoir [28], other subsurface and/or saline ecosystems [29–31]. Desulfuromonas species can catalyze oxidation of a wide range of organic compounds coupled with dissimilatory reduction of metal oxides, elemental sulfur and polysulfide [2, 30, 32]. Due to their high tolerance to saline stress, Desulfuromonas spp. have attracted attention for applicability in the saline wastewater treatment [33].

Until recent years, a few of the pure and enrichment cultures of the genus Desulfuromonas have been established with different stimulation approaches. ‘Desulfuromonas soudanensis’ WTL and Desulfuromonas sp. DDH964 were cultured from the underground Soudan iron mine through in situ enrichment with the graphite anode at + 0.24 V (vs the standard hydrogen electrode [SHE]) as electron acceptor [29]. The two strains formed a separate clade together with Desulfuromonas sp. TF that was isolated with an electrode as the acceptor [29]. The anode-stimulated strain WTL released electrons at approximately 0.1 V more positive redox potentials compared to G. sulfurreducens, while it showed slower Fe (II) generation rates when incubated with a poorly crystalline Fe (III) oxide, revealing that its extracellular respiration is tuned for high-potential electron acceptors [29]. The strain WTL survived under the unplanned oxygen exposures, due to power outage in laboratory [29], suggesting the oxygen tolerance ability of this strain. On the other hand, Desulfuromonas sp. AOP6 was anaerobically isolated from the subseafloor sediments through enrichment with the crystalline Fe (III) oxide goethite (α-FeOOH) as electron acceptor in our previous studies [34, 35]. The redox potentials of the crystalline Fe (III) oxides (less than −0.1 V vs SHE) [36] were lower than that of the constant electrode used to isolate the strain WTL (+ 0.24 V vs SHE) [29], suggesting that the crystalline Fe (III) oxide-stimulated strain could use low-potential electron acceptors. In addition, another strict anaerobic strain, Desulfuromonas acetexigens 2873T, was originally isolated from the digester sludge using acetate and elemental sulfur as the electron donor and acceptor, respectively, corresponding to the redox potential of −0.24 V vs SHE [32, 37]. This sulfur-stimulated strain possesses a single flagellum in polar and can respire the elemental sulfur [32], as well as transport electron to the anode [38], while its ability of reducing the Fe (III) oxides has not been examined. These Desulfuromonas spp. strains obtained using electron acceptors with distinct redox potentials show different physiological properties and thus they may have
distinct gene features, for instance, on extracellular electron transport, chemotaxis systems and oxygen tolerance. However, the functional traits and relationships of these phylogenetically close bacterial strains have not been studied well.

The objectives of this study were (i) to determine the genome-wide phylogenetic relationship of all the available genomes from the representative isolates in the order Desulfuromonadales (i.e., the higher taxon of the family Desulfuromonadaeaceae) and (ii) to perform comparative genome analysis to identify the different genomic signatures of the phylogenetically close Desulfuromonas spp. strains AOP6, WTL and DDH964 that were isolated using the different electron accepters, i.e., crystalline ferric iron oxide and anode.

Results & discussion

Phylogenomic position of halotolerant Fe (III) oxide-reducing bacteria in the family Desulfuromonadaeaceae

A genome-wide phylogenetic analysis of the order Desulfuromonadales was performed using concatenated amino acid sequences of 371 conserved proteins from 50 Desulfuromonadales genomes. The maximum-likelihood phylogenetic tree showed that these genomes were clearly grouped into two clades (Fig. 1). One clade consisted of the genera within the family Desulfuromonadaeaceae, i.e., Desulfuromonas, Pelobacter, Geoalkalibacter, Desulfo- musa and Malonomonas, and the two Geobacteriaceae-related genera Geothermobacter and Geopsychrobacter [39], which is defined as the Desulfuromonadaeaceae clade. The other contained the genera within the family Geobacteriaceae, i.e., Geobacter, two recently-described Geomonas [40] and Oryzomonas [41], and a single species of Pelobacter propionicus, which is defined as the Geobacteriaceae clade. The recently discovered crystalline Fe (III) oxide reducer Desulfuromonas sp. AOP6 [34, 35] was placed on the deepest branch of the Desulfuromonadaeaceae clade and formed a cluster together with the sulfur-reducing Desulfuromonas acetixegenus 2873T [32, 38]. The electrode-respiring bacteria Desulfuromonas soudanensis WTL [29], Desulfuromonas sp. DDH964 (or named as ‘Ca. Desulfuromonas biivabikowi DDH964’ [29]) and Desulfuromonas sp. TF [27] were phylogenetically close to each other and formed a cluster neighbor to the AOP6 cluster. These two clusters formed a subclade neighbor to the genus Geoalkalibacter.

Despite the phylogenetically close relationship of these Fe (III) oxide-, sulfur- and anode-stimulated bacterial strains AOP6, 2873T, WTL, DDH964 and TF, their genome sizes (3.26 to 4.40 Mb) and G + C contents (56.4 to 62.2%) were different to some extent (Table 1). Such differences appeared to be associated with the transposon expansion but not plasmid expansion in the genome. Neither plasmid-like sequence nor plasmid replication gene was found in the genomes of the 5 strains. In addition, the pairwise average nucleotide identities (ANI) among the 5 genomes were in a range from 69.9 to 72.5% (Table 2), being lower than the reported ANI cutoff values of the same species (94 ~ 95%) [42]. Meanwhile, the pairwise average amino acid identities (AAI) of them ranging from 62.7 to 66.5% (Table 2) were lower than the threshold value (i.e., 70%) to define the recently described genera Geomonas and Oryzomonas in the family Geobacteriaceae [40, 41]. These results suggested that some of these Desulfuromonas spp. strains would represent the new species in the family Desulfuromonadaeaceae.

General comparison of crystalline Fe (III) oxide- and anode-stimulated Desulfuromonas spp. strains

A phylogenetic orthology analysis using OrthoFinder [43] was performed to compare the genomes of the 5 Desulfuromonas spp. strains. Overall, 14,437 of the total 17,479 genes from the compared 5 genomes, accounting for 82.6% of the total genes, were assigned into 3423 orthologous groups (OGs) (Fig. 2). Hierarchical clustering analysis of the OGs revealed the different patterns of gene contents among the Fe (III) oxide-, sulfur-, and anode-stimulated strains (Fig. 2A). Among these OGs, 1515 OGs were identified as the core OGs, ranging from 42.1 to 54.8% of the total genetic elements of each strain (Fig. 2B). In addition, 190 and 132 OGs were shared only by the strains isolated using the inorganic electron acceptors (i.e., ferric iron oxide or sulfur) and anode, respectively (Fig. 2B). These results underscored a large genetic difference of these phylogenetically close bacterial strains.

Synteny comparisons of the complete genome sequence of the Fe (III) oxide-stimulated strain AOP6 with those of the anode-stimulated strains WTL (Fig. 3A) and DDH964 (Fig. 3B) were performed to determine the genomic difference and rearrangement between the Desulfuromonas spp. strains stimulated with different electron acceptors. From the comparisons, the Fe (III) oxide-stimulated strain AOP6 was characterized by the presence of a complete gene cluster for flagellar biosynthesis in the genome (Fig. 3). Interestingly, this gene cluster was highly conserved in the genomes of Desulfuromonadales bacteria except for 4 strains of the anode-stimulated Desulfuromonadaeaceae bacteria (i.e., ‘D. soudanensis’ WTL, Desulfuromonas sp. DDH964, Desulfuromonas sp. TF, and Geopsychrobacter electrodiphilus DSM 16401) (Fig. 1). Previous studies revealed that Geobacter species expressed flagella for chemotaxis toward Fe (III) oxides in subsurface environments to achieve the efficient respiration [21, 44]. The conservation of the flagellar biosynthetic gene cluster by the Desulfuromonadales members may have reflected their long-term association with the Fe (III)-oxide respiration, because
Fig. 1  Phylogenomic position of the 5 Desulfuromonas spp. strains in the reconstructed maximum likelihood (ML) tree based on the concatenated amino acid sequences deduced from 371 single-copy housekeeping genes. The branch length shows genetic distance with 1000 bootstrap replicates. Blue color font shows the anode-stimulated strains in the order Desulfuromonadales. Bold and normal fonts show the complete and incomplete genome sequences, respectively, of the strains. Number in brackets shows the count of deduced multiheme c-type cytochrome in the genomes. Pink circle shows the strain that had no gene cluster for flagellum biosynthesis, and the red, black and blue stars show the strains stimulated by using the crystalline Fe (III) oxide, elemental sulfur and anode as the electron acceptors, respectively.

Table 1  Desulfuromonas genomes used for comparative genomics

| Name                      | Isolation approach         | Sample type | Assembly level | GC% | Size [bp] | CDS Transposase | Reference                  | Accession     |
|---------------------------|----------------------------|-------------|----------------|-----|-----------|-----------------|----------------------------|---------------|
| Desulfuromonas sp. AOP6   | Geothite stimulated        | Cell culture| Complete       | 56.4| 3,269,909 | 3000            | Guo et al. 2020            | AP022810      |
| D. acetexigens 2873T      | Sulfur stimulated          | Cell culture| Contig         | 60.3| 3,683,125 | 3388            | Katuri et al. 2017         | GCF_900111775 |
| 'D. soudanensis WTL'      | Anode stimulated           | Cell culture| Complete       | 61.2| 3,958,620 | 3504            | Badalamenti et al. 2016    | CP010802      |
| Desulfuromonas sp. DDH964 | Anode stimulated           | Enrichment  | Complete       | 62.2| 3,924,652 | 3574            | Badalamenti, JP (unpublished)| CP015080      |
| Desulfuromonas sp. TF     | Electrode stimulated       | Enrichment  | Scaffold       | 58.6| 4,402,753 | 4013            | Kim et al. 2014            | GCF_000472285 |
Fe (III) has been thought to be abundant on the primitive Earth, potentially serving as the globally significant extracellular electron acceptor, even prior to the availability of nitrate, sulfate, and oxygen [45]. However, in the possible evolutionary history of *G. sulfurreducens*, a transposase gene was inserted into the master transcriptional regulator for the flagellar gene expression, which disrupted the flagella expression and further impaired the Fe (III) oxide reduction [44]. Therefore, we hypothesized that the lack of the flagellar biosynthetic gene cluster would trigger or enhance the Fe (III) oxide-reducing bacteria to alter the way to transfer electrons, e.g., to use alternative electron acceptors. Our comparative analysis showed that the AOP6 genome contained more chemotaxis gene clusters and specific multiheme c-type cytochrome encoding genes, whereas the WTL and DDH964 genomes possessed the operons for the *caa*3- and *cbb*3-type cytochrome c oxidases, more abundant transposable elements (TEs), and more abundant pseudogenes. Additionally, a number of genomic rearrangements identified from the comparisons (the strain AOP6 vs WTL or DDH964) were widely distributed across the whole genomes (Fig. 3). The genomic

|                | ANI | AOP6 | 2873^T | WTL | DDH964 | TF |
|----------------|-----|------|--------|-----|--------|----|
| AAI            |     | 70.4 | 70.6   | 70.1| 69.9   |    |
| 2873^T         | 63.5| *    | 72.2   | 71.6| 71.0   |    |
| WTL            | 64.7| 63.8 | *      | 72.3| 72.5   |    |
| DDH964         | 63.3| 62.8 | 65.3   | *   | 71.0   |    |
| TF             | 63.9| 62.7 | 66.5   | 64.7| *      |    |

Table 2 Pairwise similarities (%) of ANI and AAI among the five *Desulfuromonas* genomes

Fig. 2 Orthologous groups (OGs) across the genomes of the 5 *Desulfuromonas* spp. strains. A, heatmap with hierarchical clustering showing the OGs patterns of the 5 strains. B, bar plot showing the counts of OGs shared by the different strains. Red-, black- and blue-colored strain names in A and B show those strains stimulated by using the crystalline Fe (III) oxide, elemental sulfur and anode as the electron acceptors, respectively. Number above each bar in B shows the count of OGs shared by the strains. Number in brackets in B shows the count of OGs deduced in the genome of each strain. The colored dots with links in B show the orthologous groups shared by all the 5 strains (green), 4 of the strains (yellow), 3 of the strains (pink), and 2 of the strains (blue).
Fig. 3 (See legend on next page.)
response regulators as the substrates of CheA-mediated phosphorylation in chemotaxis pathways, were predicted to be on the 5 genomes. But only 6 to 11 of these genes were arranged in the chemotaxis gene clusters, being probably involved in chemotaxis signaling [49]. Meanwhile, the remainder cheY genes were located on the chromosome, thus some of them seemed to function in the two-component pathways unrelated to chemotaxis [22]. These two-component pathway-related cheY genes were detected only in the genomes of the strains AOP6 and 2873T.

A number of genes for MCPs were found in the 5 genomes; 14 in the strain AOP6, 18 in 2873T, 15 in WTL, 10 in DDH964, and 7 in TF (Table 3). MCPs have been assigned to different classes that designated 24H, 28H, 32H, 40H, 44H, and 64H (Table 3).

### Table 3 Gene counts of chemotaxis homologs in the five *Desulfuromonas* spp. genomes

| Gene Cluster | AOP6 | 2873T | WTL | DDH964 | TF |
|--------------|------|-------|-----|--------|----|
| cheA         | 4    | 5     | 3   | 4      | 3  |
| cheAY a      | 1    | 1     | 0   | 0      | 0  |
| cheB         | 3    | 3     | 4   | 4      | 6  |
| cheR         | 3    | 5     | 3   | 4      | 6  |
| cheW         | 6    | 8     | 9   | 6      | 8  |
| cheX a       | 3    | 3     | 0   | 0      | 0  |
| cheY b       | 15   | 11    | 16  | 10     | 7  |
| cheC         | 2    | 2     | 1   | 1      | 2  |
| cheD         | 3    | 2     | 2   | 2      | 2  |
| cheV         | 0    | 1     | 0   | 0      | 1  |
| mcp          | Total | 14 | 18 | 15 | 10 | 7 |

aThe specific che genes possessed by the flagellum-harboring strains AOP6 and 2873T.

bThe numbers in brackets indicate the counts of cheY genes in the major clusters shown in Fig. 4.

cThe numbers in brackets indicate the counts of chemotaxis genes in the major clusters shown in Fig. 4.

Chemotaxis system

Multiple copies of the chemotaxis genes were identified in the 5 genomes by using Microbial Signal Transduction Database (MiST) 3.0 [48]. Despite relatively small sizes of these genomes, 61 and 64 homologs to the che and mcp genes were found in the strains AOP6 and 2873T genomes, respectively, whereas the strains WTL, DDH964 and TF possessed 48 to 56 of these genes (Table 3). Most of the che genes were clustered in the genomes of the 5 strains (Fig. 4), except for the genes for chemoreceptors of the MCPs that were dispersed throughout the genomes (data not shown). The organization pattern of chemotaxis genes was consistent with those found in the genomes of *Geobacter* species [22]. There were 7, 6, 4, 4 and 5 major chemotaxis gene clusters in the AOP6, 2873T, WTL, DDH964 and TF genome, respectively. Their physical arrangements are showed in Fig. 4. In the case of the strains AOP6 and 2873T, the chemotaxis gene clusters (locus tags: AOP6_0888-0878 and BQ4888_RS06735-06680) were located on the upstream of the flagellar biosynthesis gene clusters (AOP6_0942-0890 and BQ4888_RS06745-06865). Three to 6 of the cheA and cheAY genes, encoding the autophosphorylating histidine kinase (CheA), were predicted to be on the 5 genomes (Table 3), while these were homologous to the known cheA genes in the *Geobacter* species. Previously, Tran and the colleagues reported that the multiple cheA genes in the *Geobacter* species were the paralogous genes that evolved separately, whereas each of cheA gene together with other che genes likely regulated a separate chemotaxis pathway [22]. Moreover, 10 to 21 of the cheY genes, encoding response regulators as the substrates of CheA-mediated phosphorylation in chemotaxis pathways, were predicted to be on the 5 genomes. But only 6 to 11 of these genes were arranged in the chemotaxis gene clusters, being probably involved in chemotaxis signaling [49]. Meanwhile, the remainder cheY genes were located on the chromosome, thus some of them seemed to function in the two-component pathways unrelated to chemotaxis [22]. These two-component pathway-related cheY genes were detected only in the genomes of the strains AOP6 and 2873T.

Fig. 3  Syntenic comparative genomic analysis data using the complete genome sequences of the strains AOP6, WTL and DDH964. A, the strain AOP6 versus WTL, B, the strain AOP6 versus DDH964. Red- and blue-colored strain names in A and B show those strains stimulated by using the crystalline Fe (III) oxide and anode as the electron acceptors, respectively. Symbols next to the scale show the positions of pseudogenes (black crosses), as well as those of the genes responsible for the biosyntheses of the multiheme c-type cytochromes (red dots) and transposases (grey dots), while bands next to the links show the positions of gene clusters responsible for the biosyntheses of the flagellum (yellow), chemotaxis system (magenta) and cytochrome c oxidase (green), as well as that of the prophage (sky blue). Links associated with the two genomes show homologous genes in same (red) and reverse (blue) directions. The gradient color of the link shows the identity between the amino acid sequences deduced for the homologous genes.
34H, 36H, 38H, 40H, 44H and 64H according to the number of 7-aa heptad repeats (H) in the cytoplasmic domain [50]. Among them, the MCPs of the classes 36H and 44H were well characterized in *Escherichia coli* and *Bacillus subtilis*, respectively. In the class of 36H receptors of *E. coli*, the positive stimuli increased methylation at all the responsible sites and further decreased kinase activity, whereas the class 44H receptor of *B. subtilis* had a different methylation mechanism, in which only one site was methylated and the other sites were demethylated in response to the positive stimuli, resulting in the increase in kinase activity [51]. Multiple alignments of the predicted MCPs showed that the strains AOP6 and 2873T had MCPs of the classes 36H, 38H, 40H, 44H and 64H, and the strains WTL, DDH964 and TF had those of the classes 34H, 40H, 44H and 64H. Although the anode-stimulated strains lacked the MCPs of the class 36H that were previously detected in the genomes of *Geobacter metallireducens* and *G. uraniireducens* [22], all of these 5 strains possessed the homologs of the 40H-MCP EsnA that was probably associated with the colonization on electrodes [19].

Six types of the chemotaxis clusters in the *G. sulfurreducens*, *G. metallireducens* and *G. uraniireducens* genomes were well characterized [22]. Here, the types of chemotaxis clusters found in the 5 genomes were predicted by comparing with the above-mentioned 6 types. The *E. coli*-like type 2, *Dif*-like and *Deltaproteobacteria*-specific β group clusters were found in all the genomes (Fig. 4); whereas the *Frz*-like clusters were detected in the AOP6 and 2873T genomes (Fig. 4). Though the chemotaxis clusters AOP6_0888–0878 and BQ4888_RS06735–06680 in the upstream of the flagellar clusters were similar to the β group clusters containing the
chemotaxis genes (i.e., cheA, cheW, cheB and cheR) but no mcp genes, this cluster also included other chemotaxis genes (i.e., cheC, cheD and cheX), indicating a unique type of chemotaxis cluster for the strains AOP6 and 2873T (Fig. 4A). The cheC and cheD genes, encoding the phosphatases of CheY protein (CheY-P) and the chemoreceptor glutamine deamidases, respectively, were translationally coupled and interacted to each other to regulate the chemotaxis sensing, whereas the cheX gene encoded another type of CheY-P phosphatase, i.e., CheX, that was different from the CheC [52]. Two monomers of CheX formed a homodimer as a functional unit that represented the most powerful CheC-type phosphatase [52, 53]. Due to the physically position neighbor to the flagellar clusters, it was tempting to speculate that these clusters (AOP6_0888–0878 and BQ4888_RS06735–06680) regulated the flagellar-based motility of the strains AOP6 and 2873 T. However, as the complexity of gene organization, the functional details of this cluster should be further examined by molecular approaches. Overall, compared with the anode-stimulated Desulfuromonas spp. strains, the Fe (III) oxide- and sulfur-stimulated strains AOP6 and 2873T possessed more abundant and diverse chemotaxis genes, in addition to the complete flagellar biosynthesis gene cluster, thereby enabling their possibly versatile cellular behaviors in response to environmental stimuli.

**Multiheme c-type cytochrome**

Multiheme c-type cytochromes are key for electron transport during the metal oxide and electrode respirations. To assess the diversity of the cytochromes, 28, 27, 24, 19 and 22 orthologous groups of multiheme c-type cytochromes (OGCs) were picked up from the genomes of the strains AOP6, 2873T, WTL, DDH964 and TF, respectively (43 OGCs in total). A heatmap with hierarchical clustering of these OGCs showed that the profiles of multiheme c-type cytochrome of the 5 strains were clearly grouped into two clusters; one consisted of the strains AOP6 and 2873T, while the other was comprised of the strains WTL, DDH964 and TF (Fig. 5). There were only 5 core OGCs possessed by all the five strains, i.e., the homologous genes of omcI (OGC02), ppcA (OGC03), omcQ (OGC04), imcH (OGC14) and cbcL (OGC15) characterized in the Geobacter species [10, 54]. ImcH and CbcL were identified as the inner membrane c-type cytochromes involved in the early steps of electron transfer to extracellular substrates in G. sulfurreducens, and further the two cytochromes would be required for reduction of electron acceptors with different redox potentials [55]. ImcH was for the acceptors with redox potentials higher than 0.1 V vs SHE, such as Fe (III) citrate and insoluble Mn (IV) oxides [56], while CbcL was for the low redox potential acceptors, such as the crystalline Fe (III) oxide goethite [57]. PpcA, a periplasmic c-type cytochrome, was employed in reduction of Fe (III) citrate [58], but not insoluble Fe (III) oxides and electrodes [59]. OmcI, an outer membrane c-type cytochrome, appeared to participate in reduction of Fe (III) oxide [59], while another one, i.e., OmcQ, was likely irrelevant to reduction of Fe (III) oxide [60], despite the fact that the OmcQ expression significantly increased when cultivated under a low electrode potential of −0.25 V vs SHE [61]. Although the 5 core OGCs of the c-type cytochrome genes were included in the 5 Desulfuromonas genomes in this study, the AOP6 and 2873T genomes conserved more abundant homologous genes of omcI than the other genomes. In contrast, the anode-stimulated strains seemed to possess multiple copies of imcH (2, 3 and 4 copies for the strains WTL, DDH964 and TF, respectively). Despite the presence of commonly conserved c-type cytochromes, a large
number of OGCs specific to these strains may have formed distinct electron transport ways to different electron acceptors (Fig. 5).

Previous studies revealed that the porin cytochrome conduit (Pcc) complexes acted as conduits for transporting electron across the outer membrane in the *Geobacter* species, which consisted of the periplasmic multiheme c-type cytochromes, porin-like outer membrane protein and outer membrane c-type cytochrome [62, 63]. In the case of *G. sulfurreducens*, the putative Pcc complexes *ombB-omaB-omcB* were needed for electron transport to Fe (III) oxides [59, 64]. OmcB, an outer membrane lipoprotein c-type cytochrome of the *ombB-omaB-omcB* complex, was thought to transfer electrons to the terminal reductases, such as extracellular multiheme c-type cytochromes [62, 65]. Additionally, the homolog of OmcB in *Desulfuromonas acetoxidans* was able to directly bind to the Ag electrode and promoted electron transport at the biofilm-electrode interface [66]. In this study, the *Desulfuromonas* spp. strains AOP6, DDH964 and TF possessed the complete gene cluster for the periplasmic cytochrome *c*, while the WTL and DDH964 genomes possessed the *extC* homologs (OGC13) encoding the outer membrane cytochrome *c*; however, none of the 5 strains possessed the complete *extABCD* complex gene cluster. Some of singleton genes for other periplasmic cytochrome *c* of certain Pcc complex were also found in the genomes of the 5 strains: *pecF* (OGC07) in the strains AOP6, 2873 T, WTL and DDH943, and *extK* (OGC29) in the strains AOP6 and 2873 T.

Other genes encoding the outer membrane c-type cytochromes, such as *omcO* (OGC01), *omcP* (OGC06), *omcS* (OGC08) and *omcZ* (OGC32), were found in the 5 genomes (Fig. 5). OmcO and OmcP appeared to be irrelevant to Fe (III) oxide reduction by *G. sulfurreducens* [59], but their homologs were relevant to that by *G. metallireducens* [67], indicating that these c-type cytochromes may have played different functional roles in these species. In this study, the anode-stimulated strains WTL and DDH964 possessed multiple copies of the *omcO* and *omcP* genes, but the ferric iron oxide-stimulated AOP6 possessed only one copy of *omcP*. Meanwhile, OmcS required for both Fe (III) oxide reduction [65] and electrode respiration [68] bound to e-pili as catalyzing the terminal electron transfer [69]. Recent studies showed that OmcS also formed the cytochrome-based filaments capable of transporting electron for long distances over micrometers [14, 15]. Here, multiple homologs of OmcS were found in the strains AOP6, 2873 T and TF, but not WTL and DDH964. Further, OmcZ was reported to participate in electron transport to anode with thick biofilm (>50 μm) [70], but not to anode with thin biofilm and Fe (III) oxides by *G. sulfurreducens* [59, 68]. Only one gene copy encoding the OmcZ homolog was found in the 2873 T genome.

Although the strains WTL and DDH964 did not have the homologs of OmcS and OmcZ, they possessed *pgcA* (OGC23) and *gscA* (OGC30) genes encoding extracellular multiheme c-type cytochromes that can serve as terminal reductases [71, 72]. PgcA, a soluble c-type cytochrome, was secreted to environments as electron shuttle [71]. The *G. sulfurreducens* mutants with deletion of *pgcA* lost the ability to transfer electrons to the Fe (III) and Mn (IV) oxides, but the same mutants maintained the electrode respiration capability [20], suggesting the enhancement of the metal reductions rather than electrode respiration by PgcA. However, the homolog of PgcA was not found in the genomes of the Fe (III) oxide-stimulated strain AOP6 and sulfide-stimulated strain 2873 T, but found in the anode-stimulated strains WTL and DDH964. In this context, this gene was also present in the genomes of *G. anodireducens* SD-1 and *G. sulfurreducens* YM18 that were isolated from an anode biofilm and generated high current densities in bioelectrochemical systems [72, 73].

Menaquinol:ferricytochrome c oxidoreductase (Cbc) complexes, consisting of periplasmic c-type cytochromes, b-type cytochromes, membrane proteins and other c-type cytochromes, were also associated with reduction of the Fe (III) and Mn (IV) oxides in the *Geobacter* species [59]. In this study, the genes *cbCA* (OGC17), *cbCM* (OGC21), *cbCN* (OGC24), *cbCR* (OGC25), *cbCS* (OGC19), and *cbEX* (OGC20) encoding the c-type cytochromes of Cbc complexes were found in the 5 genomes (Fig. 5). Reportedly, the expressions of the *cbCA*, *cbCN* and *cbEX* genes for the periplasmic c-type cytochromes were up-regulated in *G. sulfurreducens* with Fe (III) oxide versus Fe (III) citrate [59]. It was noteworthy that only the Fe (III) oxide-stimulated strain AOP6 possessed all of these three genes.

A number of other genes encoding c-type cytochromes found in the 5 genomes were reported to be significantly up-regulated when Fe (III) oxide was used as electron acceptor, which includes the *mrFA* (OGC09), *mrFH* (OGC26) genes in *G. sulfurreducens* [59], as well as the unnamed genes Gmet_1867 (OGC16), Gmet_0142 (OGC18) and Gmet_0679 (OGC27) in *G. metallireducens* [67]. However, no further evidence can support an association between the arrangements of these genes and the Fe (III) oxide reduction activity. In addition, the
other genes for c-type cytochromes (OGC31 and OGC33–43) were also present in some of the 5 genomes (Fig. 5). The homologs of these genes were almost found in the genomes of the Desulfuromonas species, but absent in those of the Geobacter species (data not shown). Their roles in extracellular electron transport warrant the future study. Consequently, our analysis indicated that the multi-theme c-type cytochrome profiles were obviously different among the phylogenetically close Desulfuromonas spp. strains. The Fe (III)-oxide stimulated strains possessed more abundant and diverse c-type cytochromes than the anode-stimulated strains. Particularly, the periplasmic and outer membrane cytochromes represented strain- and/or cluster-specific patterns, which may have linked with electron transports to the iron (III) oxide and anode with different redox potentials.

Cytochrome oxidase and ROS detoxifying enzyme

Although most of members in the families Geobacteraeaceae and Desulfuromonadaceae are strictly anaerobic [37, 74], some of them show the ability to detoxify reactive oxygen species (ROS) and resist low concentrations (e.g., ~10%) of oxygen [27, 29, 75, 76]. G. sulphurreducens sigma factor RpoS contributed to survival in stationary phase and upon oxygen exposure [75]. RpoS activated the cca3-type cytochrome c oxidase operon, which encodes a heme-copper terminal oxidase for the G. sulphurreducens growth using oxygen as an electron acceptor [77]. The cca3-type oxidase encoded by coxABCD was found in the genomes of G. sulphurreducens [10], G. metallireducens [54], Geomonas bemidjiensis (previously named as Geobacter bemidjiensis) [40, 78], and Desulfuromonas sp. TF [27]. On the other hand, the cbb3-type cytochrome c oxidase (CcoNOPQ) with high affinity for oxygen was expressed under low oxygen tension conditions [79], and the genes encoding this oxidase were conserved in the genomes of Geom. bemidjiensis and Desulfuromonas sp. TF [27, 78].

Intriguingly, in this study, the gene sets for these two types of oxidases were present in the genomes of the strains WTL, DDH964 and TF, but absent in those of the strains AOP6 and 2873T except for the homologous genes of ccoP that encodes the diheme cytochrome c subunit (subunit III) of cbb3-type oxidase (Table 4). Moreover, the anode-stimulated strains possessed multiple homologs of CcoP: 6 in the strain WTL, 4 in DDH964, and 4 in TF. Pseudomonas aeruginosa, a ubiquitous opportunistic human pathogen, expressed multiple cbb3-type cytochrome c oxidase isoforms by combinations of multiple isosubunits, and the strains carrying these isoforms can resist to respiratory inhibitors such as nitrite and cyanide at low concentrations of oxygen [80]. Despite only one ccoN gene encoding the catalytic subunit possessed by the anode-stimulated strains, there was still possibility that they produced 4 to 6 cbb3 isoforms by combinations with different CcoP isosubunits. Such multiple ccb3 isoforms may have brought a diverse array for oxidative stress responses for the anode-stimulated strains. Additionally, the cydAB genes encoding cytochrome bd quinol oxidase complex was found in the 5 genomes (Table 4), which is related to the provision of the trace oxygen tolerance [27]. Genes involved in the ROS detoxification, e.g., the genes for ruberythrin (rbr), rubredoxin (rub), desulfoferredoxin (dfs), and cytochrome c peroxidase (macA), abundantly existed in the 5 genomes (Table 4). It was reported that these genes were significantly expressed in G. uranireducens exposed to 5% oxygen revealed by microarray analysis [81]. Overall, comparative genome analysis showed that the cca3- and cbb3-type cytochrome c oxidases appeared to be specific to the anode-stimulated strains WTL, DDH964 and TF. They also possessed other diverse genes involved in oxygen resistance and ROS detoxification. These genetic features would confer high oxygen resistance in the strictly anaerobic bacteria, contributing to the niche differentiation of the phylogenetically close Desulfuromonas spp. strains. Reportedly, the anode-stimulated strain WTL originally inhabited in the anoxic (but not strictly anaerobic) environments at the Soudan Iron Mine borehole and it was able to survive under the unplanned oxygen exposures, due to power outage in laboratory [29], which partially supports the genomic inference.

Transposable element

Transposable elements (TEs) are usually found in the genomes of bacterial symbionts, which initially undergo massive expansion and then loss accompanied by gene inactivation and decay, genome rearrangement and genome reduction [82–85]. The large genome rearrangement and deletions associated with insertion sequence (IS) expansion enabled the symbiotic bacterium to combat host defenses by changing surface antigens and regulatory circuitry [86, 87]. Similar patterns of this genome evolution were detected in cases of some niche-restricted prokaryotes such as Sulfolobus solfataricus and certain Cyanobacteria species [88–90].

The anode-stimulated strains WTL and DDH964 possessed large numbers of TEs, particularly transposase-encoding genes that accounted for 2.2 and 3.1% of the total coding sequences in the genomes, respectively, whereas the Fe (III) oxide-stimulated strain AOP6 only contains 10 of these genes in the genome (0.3% of the total) (Fig. 3). In addition, most of the transposase-encoding genes were located nearby the pseudogenes in the WTL and DDH964 genomes (Fig. 3), suggesting that the repeated insertion-deletion of transposons induced
Table 4 Genes encoding cytochrome c oxidases, cytochrome bd oxidase, and ROS detoxifying enzymes in the five *Desulfuromonas* genomes

| Gene     | Product                                                                 | Locus tag in the genomes of *Desulfuromonas* spp. strains |
|----------|-------------------------------------------------------------------------|--------------------------------------------------------|
| sco      | cytochrome c oxidase synthesis factor                                   | AOP6: 2873, 2874                                       |
| coxA     | cytochrome c oxidase, subunit I                                        | DSOUD_0978, DBW_3007                                   |
| coxB     | cytochrome c oxidase, subunit II                                       | DSOUD_0979, DBW_3008                                   |
| coxC     | cytochrome c oxidase, subunit III                                      | DSOUD_0980, DBW_3009                                   |
| coxD     | cytochrome c oxidase, subunit IV                                       | DSOUD_0981, DBW_3010                                   |
| ctaB     | Proteheme IX farnesyltransferase                                       | AOP6_2408, BQ4888_RS16015                              |
| dfx      | Desulfoferredoxin (superoxide reductase)                               | AOP6_2409, BQ4888_RS16020                              |
| ccoN     | cytochrome c oxidase, subunit I                                        | DSOUD_2311, DBW_2371                                   |
| ccoO     | cytochrome c oxidase, subunit II                                       | DSOUD_2312, DBW_2372                                   |
| ccoP     | cytochrome c oxidase, subunit III                                      | DSOUD_2313, DBW_2373                                   |
| ccoQ     | cytochrome c oxidase assembly chaperone                                 | AOP6_0637, BQ4888_RS14340                              |
| ccoS     | cytochrome c oxidase assembly protein CcoS                              | AOP6_0462, AOP6_1193                                  |
| cbb3     | cytochrome c oxidase, subunit III                                      | DSOUD_0601, DBW_0838                                   |
| rbr      | Rubrethrin                                                             | AOP6_0339, BQ4888_RS13615                              |
| sodA     | Superoxide dismutase                                                   | AOP6_0358, BQ4888_RS09810                              |
| macA     | Cytochrome c peroxidase                                                 | AOP6_0314, AOP6_0558                                  |

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the loss of some of functional genes, such as those encoding the transcriptionsal regulator, RNA methyltransferase, ATPase, polysulfide reductase and methyl-accepting chemotaxis protein (data not shown). Meanwhile, 31 and 6 transposase-encoding genes were also found in the 2873 T and TF genomes; however, due to the highly fragmented assemblies of the strains 2873 T and TF (consisting of 41 and 167 contigs, respectively), the absolute counts of TEs would be underestimated [84]. Thus, these results only suggest an expansion of TEs in the genomes of the strains WTL and DDH964.

In order to better understand the pattern of TEs, the predicted transposase genes were classified by IS family. This analysis showed that the transposase genes in the WTL genome were mainly affiliated to families IS1595, IS3 and IS4, while those in the DDH964 genome were predominately from families IS256, IS4, IS200/IS605, IS21, IS110, IS3, and others (Fig. 6). The distinct profiles of IS family between the two genomes suggested that these anode-stimulated strains may have independently undergone the TE expansions. Our analysis identified the abundant and diverse transposons spread across the genomes of the anode-stimulated WTL and DDH964 (Figs. 3 and 6). Other important features were (i) the absence of the gene cluster for flagellar biosynthesis (see section 2) and (ii) the presence of diverse genes involved in oxygen resistance and ROS detoxification (see section 5) in these two genomes. We speculated that, when the flagellar gene cluster is lost from the Desulfuromonas genome, the bacterial mobility would be decreased and the cells would be restricted in specific habitats with relatively constant conditions, such as existing simple types of electron acceptors. This situation may trigger the transposon-mediated genome rearrangement that results in the selection of the essential genes for this specific habitat and the elimination of other unnecessary genes from the genome. However, the genome sizes of the strains WTL and DDH964 are larger than those of the strains AOP6 and 2873 T, indicating the gene acquisition and enrichment may have also occurred during the genome rearrangement. It is likely that the decrease in mobility also affects the ability of escaping from the abrupt change in environment conditions, for example the exposure to oxygen, so that the genes involved in stress resistance would be gained and enriched in the genome. Interestingly, it was reported that the genes involved in the formation of pili and flagella, as well as chemotaxis sensory regulators, in G. uraniireducens were significantly down-regulated under oxidative stress conditions, while those for oxygen resistant enzymes were up-regulated under the same conditions [81], implying the opposite relationship between the expression of these genes. This evolutionary scenario partially explained that the strains AOP6 and 2873 T possessed more numerous numbers and types of the genes involved in chemotaxis and c-type cytochrome, while the strains WTL and DDH964 had more diverse genes for oxygen resistance.

Conclusions
In this study, we carried out comparative genome analysis to identify the different genomic signatures between ferric iron oxide- and anode-stimulated Desulfuromonas spp. strains. The results indicated that the compared 5 genomes possessed distinct gene sets responsible for flagellar biosynthesis, chemotaxis, electron transfer, oxygen resistance and genome rearrangement. Particularly, the crystalline Fe (III) oxide-stimulated strain AOP6 would exhibit the flagellum-based mobility with more diverse chemotaxis sensory systems and more abundant c-type cytochromes for reduction of electron acceptors with low redox potentials. On the other hand, the anode-
stimulated strains WTL and DDH964 lacking the flagellum-based mobility likely weakened the ability to use the low redox potential electron acceptors but enabled to survive under oxygen exposure. Moreover, the transposon expansions would mediate the genome rearrangements in the strains WTL and DDH964 genomes. These findings cast light on genome evolution of the phylogenetically close Desulfuromonas spp. strains that are involved in metal reduction in subsurface environments.

Methods

Genome-wide phylogeny of the order Desulfuromonadales

In order to perform a genome-wide phylogenetic analysis, all the available genomes (50 genomes in total) affiliated with the order Desulfuromonadales were obtained from GenBank/DDJ/EMBL on March 11, 2020. Single copy marker genes were inferred using OrthoFinder v2.3.3 [91], and the amino acid sequences deduced from these genes were used to perform a multilocus sequence analysis as previously reported [84, 85], with minor modifications. In brief, multiple sequences alignments were inferred for each gene using MAFFT v7.455 [92] and concatenated for phylogenetic analysis. An approximately-maximum-likelihood phylogenetic tree was generated using FastTree v2.1.10 with the Gamma20 model [93]. FigTree v1.4.4 (https://github.com/rambaut/figtree) was used for the visualization of the tree.

Comparative genomics

In an attempt to investigate the genomic features for the closely related Desulfuromonas spp. strains with different enrichment approaches, the complete genome sequences of the strains AOP6, WTL, and DDH964, as well as the draft sequences of strains 2873T and TF, were selected of the strains AOP6, WTL, and DDH964, as well as the enrichment approaches, the complete genome sequences closely related Desulfuromonas spp. strains with different

Abbreviations

AAI: Average Amino acid Identity; ANI: Average Nucleotide Identity; MCP: Methyl-accepting Chemotaxis Protein; OG: Orthologous Group; OGC: Orthologous Group of c-type Cytochrome; ROS: Reactive Oxygen Species; TE: Transposable Element

Supplementary Information

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Additional file 1.

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Authors’ contributions

YG, TA and TH contributed to the conception, investigation and design of the study. YG performed the bioinformatic analyses and wrote the draft manuscript. TA and TH contributed to acquire the funding, discuss the results, and revise the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
All data analyzed in this study are available through NCBI GeneBank and ReSeq databases, are accessible through the accession numbers listed in Table 1 for the major data and in Table S1 for all the data, respectively.

Declarations

Ethics approval, accordance 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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