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RESEARCH ARTICLE

Long-term adaptive evolution of *Shewanella oneidensis* MR-1 for establishment of high concentration Cr(VI) tolerance

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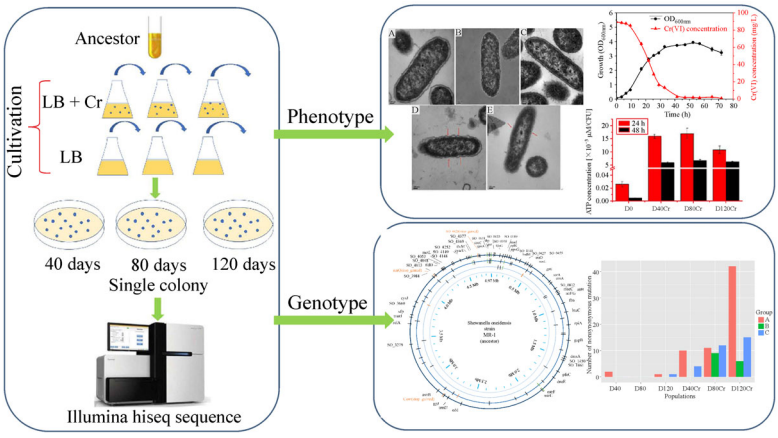
HIGHLIGHTS

- *Shewanella oneidensis* MR-1 was acclimated to grow with Cr(VI) of 190 mg/L.
- Whole genomes from 7 populations at different acclimation stages were sequenced.
- Gene mutations mainly related to efflux pumps and transporters.
- An adaptation mechanism of MR-1 to high concentration of Cr(VI) was proposed.

GRAPHIC ABSTRACT



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ABSTRACT

Acclimation is the main method to enhance the productivity of microorganisms in environmental biotechnology, but it remains uncertain how microorganisms acquire resistance to high concentrations of pollutants during long-term acclimation. *Shewanella oneidensis* MR-1 was acclimated for 120 days with increasing hexavalent chromium (Cr(VI)) concentrations from 10 to 190 mg/L. The bacterium was able to survive from the highly toxic Cr(VI) environment due to its enhanced capability to reduce Cr(VI) and the increased cell membrane surface. We sequenced 19 complete genomes from 7 populations of MR-1, including the ancestral strain, the evolved strains in Cr(VI) environment on days 40, 80 and 120 and their corresponding controls. A total of 27, 49 and 90 single nucleotide polymorphisms were found in the Cr(VI)-evolved populations on days 40, 80 and 120, respectively. Nonsynonymous substitutions were clustered according to gene functions, and the gene mutations related to integral components of the membrane, including efflux pumps and transporters, were the key determinants of chromate resistance. In addition, MR-1 strengthened the detoxification of Cr(VI) through gene variations involved in adenosine triphosphate binding, electron carrier activity, signal transduction and DNA repair. Our results provide an in-depth analysis of how Cr(VI) resistance of *S. oneidensis* MR-1 is improved by acclimation, as well as a genetic understanding of the impact of long-term exposure of microorganisms to pollution.

1 Introduction

Environmental biotechnology is one of the most important methods for the remediation of various polluted environments (Rittmann et al., 2006; Liu et al., 2018), for instance wastewater treatment. However, most natural microorganisms can only grow in environments with low concentrations of pollutants and usually transform pollutants with low efficiency. People therefore culture microbial consortia with gradually increased pollutants to enhance their resistance and transforming ability to pollutants (Carlson et al., 2019), which is designated as the acclimation process. Understanding how microorganisms respond to

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pollutant stress during the acclimation process is of vital importance to regulate the microbial consortia and enhance the performance of bioremediation.

Several studies have studied the short-term effects of pollutants on microorganisms at the mRNA and protein levels (Hou et al., 2018; Gang et al., 2019). For instance, Wang et al. used two-dimensional electrophoresis technology to analyze the effect of chromium (Cr, atomic weight of 52) on membrane cytochromes in *Shewanella oneidensis* MR-1 wild type and its mutant (Wang et al., 2014). Chourey et al. assessed the biological impact, in terms of the differences of mRNA and protein expression patterns on *S. oneidensis* MR-1 during 24-h exposure to hexavalent chromium (Cr(VI)) (Chourey et al., 2006). Brown et al. described temporal alterations in mRNA expression and protein synthesis in response to acute, toxic levels of Cr(VI) to understand the potential molecular mechanisms of resistance to the metal (Brown et al., 2006). Other researchers have investigated the evolution of bacteria in the laboratory environments without contaminant pressure. For example, Lenski et al. evaluated the evolution of *Escherichia coli* in a laboratory environment during long-term cultivation (Lenski et al., 1991; Barrick et al., 2009; Blount et al., 2012). Unlike the slow evolution in a natural or slightly modified natural environment, microorganisms in environmental engineering systems must evolve rapidly to adapt and efficiently transform pollutants to survive from the quickly changing environment. However, this acclimation mechanism of specific microorganism to increasing concentrations of pollutants in environmental engineering systems has barely been mentioned in previous studies.

Heavy metals are major pollutants in many environments, such as groundwater, soil, and river basin (Hu et al., 2014; Liang et al., 2019). These metals also contaminate drinking water, agricultural crops, and aquatic foods, and drinking contaminated water or consuming polluted foods can cause serious damage to human health (Li et al., 2014; He et al., 2019). Though Cr is an essential trace element for living organisms, Cr(VI), which can enter the cell through the sulfate transport channel of the cell membrane and change the genetic traits of microorganisms, is highly toxic (Petrilli and De Flora, 1977) and mutagenic (Nishioka, 1975) to humans. One common environmental treatment of Cr(VI) is transforming Cr(VI) to trivalent chromium (Cr(III)), which is considerably less harmful to humans (Lytle et al., 1998). *S. oneidensis* MR-1, a facultative aerobic and Gram-negative bacterium, can reduce Cr(VI) to Cr(III) and therefore can be used for the bioremediation of Cr(VI) contamination. However, the concentration of Cr (VI) in certain cases may be higher than hundreds of milligrams per liter, which will suppress the growth of *S. oneidensis* MR-1 and the reduction of Cr(VI).

Whole genome sequencing combined with long-term acclimation to pollutants has become a highly important technique to explore the adaptive process of microorganisms (Conrad et al., 2011; Dettman et al., 2012). A pure culture of a bacterium has a relatively small genome, and using modern sequencing technologies, it is easy to find the variations between the evolved and the original strains to infer the adaptive mechanism during the acclimation process. In the present study, the associations between mutations and chromate resistance were investigated by developing evolved strains, which were cultured with increasing concentrations of Cr(VI). The objective of this study is to obtain strains with enhanced chromate resistance and to gain insight into the gene evolution and resistance mechanism of *S. oneidensis* MR-1 in a 120-day cultivation with increasing Cr(VI) exposure compared to the control populations with no added Cr(VI). Interestingly, *S. oneidensis* MR-1 exhibited improved resistance to chromate and reached a limitation of Cr(VI) reduction simultaneously. Genome re-sequencing revealed that mutations occurred in different stages of the acclimation process. The results of our study suggest that an integral component of the membrane defense system is a primary adaptation strategy for *S. oneidensis* MR-1 copping with high concentrations of Cr(VI).

2 Materials and methods

2.1 Bacterial strains and culture conditions

The *S. oneidensis* strain MR-1 was inoculated in Luria-Bertani (LB) broth (Venkateswaran et al., 1999) (pH 7.2) and incubated at 30°C by shaking at 150 r/min in an incubator under aerobic conditions. The LB medium consists of tryptone (10 g/L), yeast extract (5 g/L), and NaCl (10 g/L). A starter culture was derived from a single colony on a freshly streaked LB plate. The colony was then transferred into 100 mL of LB broth in a 250 mL Erlenmeyer flask and incubated for 16 h. This culture was regarded as the original population (designated D0).

2.2 Long-term acclimation experiment

Six replicates derived from the same colony of the original *S. oneidensis* strain MR-1 were used for long-term acclimation experiments. Three replicates served as control and were grown in LB broth without Cr. These strains were collected at days 40, 80 and 120 and designated as populations D40, D80 and D120, respectively. The other three replicates were inoculated in LB broth with increasing Cr(VI). The time and corresponding concentration of Cr(VI) are shown in Table S1. The strains collected at days 40, 80 and 120 were designated as populations D40Cr, D80Cr and D120Cr, respectively. The Cr(VI) concentrations of each population at days 40, 80 and 120 were 90, 140 and 190 mg/L, respectively. For the

acclimation process, 0.5 mL of cells at stationary phase (48±0.5 h) were transferred into 49.5 mL fresh LB broth amended with potassium chromate (K_2CrO_4, Sigma-Aldrich, St. Louis, MO, USA) in 100 mL Erlenmeyer flasks with a dilution of 1/100, giving an initial optical density at 600 nm of 0.06. After every 48 h of propagation, samples were transferred into fresh LB broth with 25% glycerol and stored at −80°C.

2.3 Growth curve and Cr(VI) reduction efficiency

Growth curve experiments and evaluations of reduction efficiency were performed by measuring the optical density at 600 and 540 nm of triplicate cultures over several time points using a Spectronic 20D + spectrophotometer. Evolved populations D40Cr, D80Cr, and D120Cr were inoculated in Cr(VI)-containing LB medium, and populations D40, D80, and D120 were grown in LB medium without Cr(VI) as controls. Cr(VI) in the supernatant was measured using the 1,5-diphenylcarbazide method (Park et al., 2000) at a wavelength of 540 nm. Uninoculated LB medium containing Cr(VI) served as abiotic control.

2.4 DNA sequencing

Whole genome re-sequencing is a powerful tool to identify mutations accumulated during long-term acclimation processes (Brockhurst et al., 2011). The populations of the ancestral strain i.e. D0 and 18 evolved strains that evolved for 40, 80 and 120 days in their respective treatments were streaked onto LB agar plates. Randomly-selected colonies from each sub-strain were cultured for 16 h to extract DNA. The DNA paired-end sequencing was performed on a HiSeq 4000 Sequencing Sytem (Illumina, San Diego, CA, USA) in Majorbio (Shanghai, China).

2.5 Bioinformatics analysis process

All high-quality reads were mapped with the reference genome of *S. oneidensis* MR-1 (NC_004347.2) (Heidelberg et al., 2002) downloaded from the NCBI website using Burrows-Wheeler aligner BWA (Li and Durbin, 2009). The sequence alignment map (SAM) format of files was converted to sorted and indexed binary alignment map (BAM) files using SAMtools version 1.6. In the process of preparing the library, artifacts may be introduced from over-amplification during PCR. Duplicate reads were removed by the Picard tool. We used Genome Analysis Toolkit (GATK; version 3.8-0) (McKenna et al., 2010) and SAMtools (Li et al., 2009) to identify all mutations in the evolved strains compared to the ancestral strain, including single-nucleotide polymorphisms (SNPs) and deletion-insertion polymorphisms (DIPs). BAM files were re-aligned with the GATK IndelRealigner, and then variations were called by the GATK HaplotypeCaller. We also used the SAMtools 'mpileup' command to identify SNPs and insertions/deletions (indels) and then screened out the common results of these two tools. VariantFiltration of GATK was used to filter out low-quality SNPs and indels by hard criteria, 'QD < 20.0 || ReadPosRankSum < −8.0 || FS>10.0 || QUAL < $MEANQUAL'. We then extracted the mutation sites marked as PASS in the vcf file and separated SNPs and indels using the 'SelectVariants' command. To obtain credible variation information, we filtered the SNPs and indels once again by hard filter criteria implemented in the commands, 'QD < 2.0 || FS>60.0 || MQ < 40.0 || MQRankSum < −12.5 || ReadPosRankSum < −8.0' and 'QD < 2.0 || FS>200.0 || ReadPosRankSum < −20.0', respectively.

3 Results and discussion

3.1 Enhanced chromate resistance and phenotypic differences

During the acclimation process, Cr(VI) concentration in the culture was gradually increased, and *S. oneidensis* MR-1 was forced to adapt to the changing environment. The ancestral D0 cells and the evolved clones D40Cr, D80Cr and D120Cr exhibited different resistance and reduction efficiencies to Cr(VI) (Fig. 1). The ancestral D0 cells displayed substantial growth in LB broth without Cr(VI) (Fig. 1A, maximum OD_{600} of 6.69) but did not grow in LB broth containing Cr(VI) of 90 mg/L (inset in Fig. 1A). However, the evolved populations D40Cr, D80Cr and D120Cr grew well in Cr(VI)-containing LB broth with maximum OD_{600} values of about 4.10, 3.64, and 3.53, respectively. This result indicated that the MR-1 strains evolved a strong Cr(VI) resistance during the acclimation process and the Cr(VI) resistance of MR-1 could be continuously enhanced.

The other three control populations, i.e. populations D40, D80 and D120 which were cultured in LB broth without Cr(VI), showed no significant improvement in Cr(VI) tolerance or reduction (Fig. S1). However, 104 mg/L Cr(VI) (Fig. 1B) was 99.99% reduced by the evolved population D40Cr, which had been acclimated for 40 days in a Cr(VI) environment. After continuous acclimation, the evolved strains at days 80 and 120 reduced Cr(VI) of 140 mg/L and 190 mg/L to Cr(III) with efficiencies of 68.55% and 47.70%, respectively (Figs. 1C and 1D). Although population D120Cr was able to resist 190 mg/L of Cr(VI), this population reduced less than half of Cr(VI) in the medium. When D120Cr was inoculated in a medium with doubled amounts of yeast extract (10 g/L) and tryptone (20 g/L), the culture achieved a Cr(VI) reduction efficiency of 86.36% (Fig. S2), which means that the nutrients in 50-mL LB broth are not enough to support MR-1 fully reducing the Cr(VI) of 190 mg/L. These results indicated that besides the enhanced resistance to Cr(VI), MR-1 strains

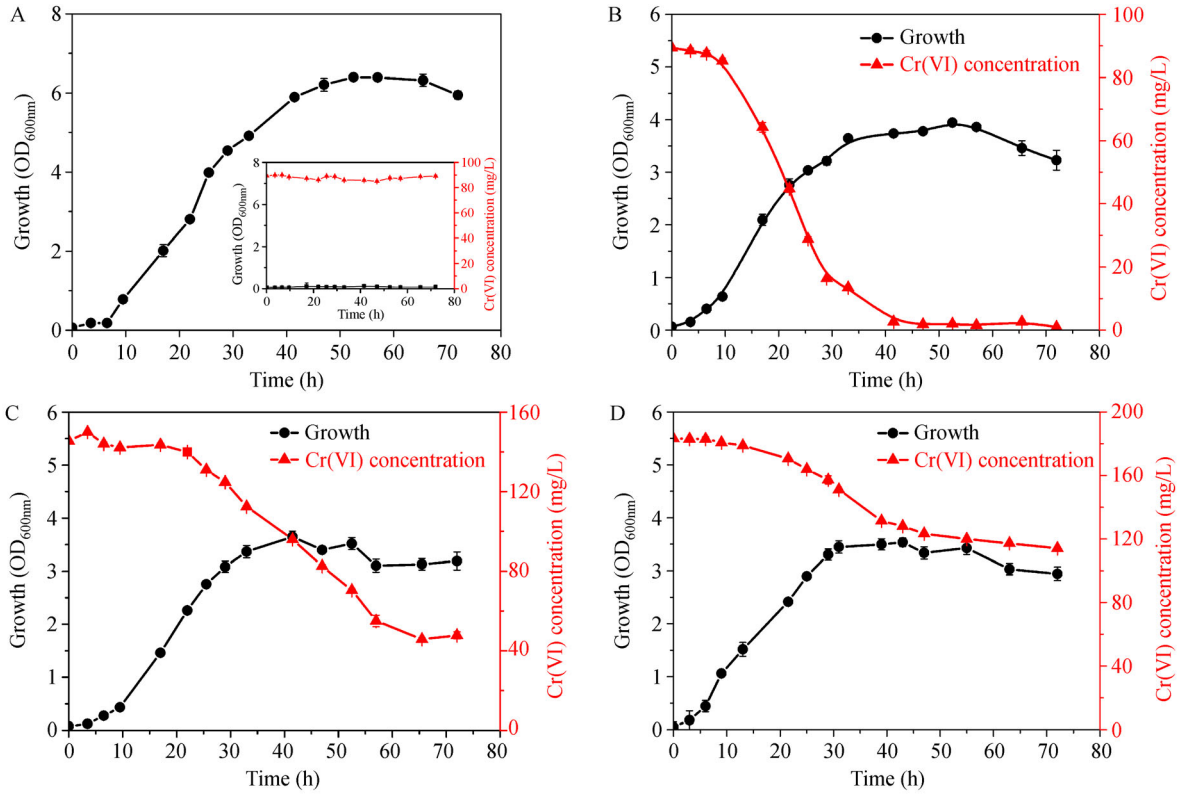

Fig. 1 Growth rate (black line) and reduction kinetics (red line) of ancestral and evolved *S. oneidensis* MR-1 strains in different initial concentrations of hexavalent chromium Cr(VI). (A) The growth curves of ancestral strain D0 cultured in Luria-Bertani (LB) broth without Cr(VI) and with 90mg/L Cr(VI) (inset). (B) Evolved strain at day 40 (population D40Cr) was inoculated in LB broth with 90 mg/L Cr(VI). (C) Evolved strain at day 80 (population D80Cr) was inoculated in LB broth with 140 mg/L Cr(VI). (D) Evolved strain at day 120 (population D120Cr) was inoculated to LB broth with 190 mg/L Cr(VI).

also continuously evolved to enhance their ability to reduce a high amount of Cr(VI) to Cr(III) during the acclimation process, even though there were not enough nutrients.

The phenotypic difference between evolved and ancestral strains was also reflected in the change of the outer cell membrane, which was revealed by transmission electron microscopy (Fig. S3). Compared with the ancestral D0, we observed more pleats on the cell surface of MR-1 strains that had evolved in Cr(VI) for 80 or 120 days (Figs. S3D and S3E). While adsorption is a major mechanism of Cr (VI) detoxification to cells (Cheung and Gu, 2007), the increase of pleats on the cell surface markedly increased the membrane area, facilitated the adsorption of Cr(VI) and thus increased the adaptability of bacteria to heavy metals in the environment.

3.2 Gene mutations accumulated during long-term acclimation

Long-term acclimation of MR-1 in different Cr(VI) conditions induced remarkable differences between the evolved mutants of MR-1 and the ancestral strain (Figs. 1 and S1). Whole genome re-sequencing was used to reveal how the evolved mutants gained such a high resistance and quickly reduced Cr(VI). The GATK tool was used to find SNPs in whole genomes of the 19 strain samples by aligning the reference genome of *S. oneidensis* MR-1 downloaded from Ensembl website (ASM14616v2, www.ensembl.org).

The bioinformatics analysis suggested that the numbers of spontaneous mutations in populations D40, D80, or D120 were less than 2, due to the absence of an external selective pressure (Fig. 2). This result indicated that the mutation rate of bacteria in the control environment for 120 days was very slow. Mutations occurred in sites relating to genes SO_0719, *gltX*, and SO_3337 in population D120 and genes SO_0012, *tldD*, *lys* and *macB* in population D40, which are mainly related to transporter and metabolic activities (Table S2–S6).

More mutations were found in the MR-1 strains evolved in the Cr(VI) environment, i.e., populations D40Cr, D80Cr and D120Cr, and the numbers of SNPs increased along with the acclimation process due to the increasing exposure pressure of chromium. A total of 90 SNPs was found from all replicates in population D120Cr, whereas 49 and 27 mutation points were found from populations D80Cr and D40Cr, respectively. Furthermore, the rapid adaptation to Cr conditions may be facilitated by the

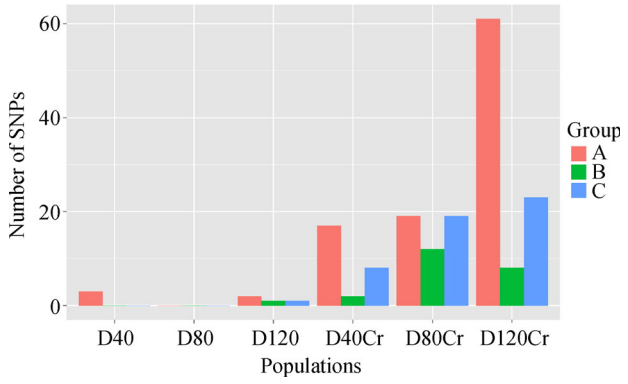

Fig. 2 Number of SNP mutations in 6 evolved populations. Populations D40, D80 and D120 were strains derived from ancestral MR-1 and cultured in LB medium without chromium for 40, 80 and 120 days, respectively. Populations D40Cr, D80Cr and D120Cr were evolved MR-1 strains cultured for 40, 80 and 120 days in LB broth with increasing levels of hexavalent chromium.

elevated spontaneous mutation rate during the long-term Cr(VI) acclimation. These findings are in line with Gerrish & Lenski (Gerrish and Lenski, 1998) who reported that higher mutation rates can lead to increased rates of fitness. The accumulation of mutations enhanced the ability of the bacterium to adapt to the increasing levels of Cr(VI) in the environment.

Aside from the mutation number, we also need to know the function and metabolic pathways involved in the altered genes to better understand the effect of gene variation on cell metabolism. The functional enrichment analysis was performed in DAVID 6.8 (Huang et al., 2009a,b) website online. We mainly carried out the Gene Ontology (GO) function of the annotation analysis on three categories including biological process, cellular component and molecular function.

The most enriched GO Terms of population D120Cr related to cell wall organization, peptidoglycan biosynthesis and regulation of cell shape in the biological process category (Fig. 3). The long-term exposure to Cr(VI) environment enhanced the Cr resistance of MR-1, which is partially ascribed to the gene variations related to the cell membrane. As Cr concentration in medium was increased, microorganisms have to enhance the function of cell membrane to form a protective shell to maintain the normal morphology, which corresponds with the result of functional enrichment in the biological process category. Genes including *murJ*, *murI*, *ftsW*, *murG*, *murC*, *murE*, SO_3735, *murF*, *mraY*, *murD*, *uppP* were mutated genes that are involved in cell wall function and cell shape. For example, gene *murJ* relates to peptidoglycan lipid II flippase which contributes to enhanced current-generating capability (Kouzuma et al., 2014), and *murI* is crucial to detoxification and repair of membrane damage during exposure to U (VI) (Orellana et al., 2014) and acute chromate (Chourey et al., 2006). SO_3735 participates in peptidoglycan biosynthesis and responds to environmental pressure like cold temperature (Zeng et al., 2016). The bacterium can take up Cr(VI) through the sulfate transport channels on cell membrane (Cervantes and Silver, 1992). Aguilera et al. (Aguilera et al., 2004) reported that the resistance of Cr(VI) is attributed to decreased uptake or enhanced efflux of Cr (VI) by the cell membrane. There are 6 genes (SO_4087, SO_4088, *natB*, SO_4090, *macC*, *nosY*) that are involved in efflux transmembrane transporter activity. The toxic substances that enter into the cell through the membrane sulfate transport channels need to be excreted out under the transporter activity.

During the reduction process, Cr(VI) as the electron acceptor is reduced to Cr(III). Higher concentrations of Cr (VI) requires more electrons to accomplish the reduction, and mutations were therefore observed on 29 genes involving in the electron transport process (Fig. 3). In addition, 12 genes relating to anaerobic nitrite reduction were mutated, which is consistent with the finding that chromate and nitrite interactions in *S. oneidensis* MR-1 plays a part in Cr(VI) reduction (Viamajala et al., 2002).

The GO terms analysis in population D120Cr suggested that to adapt to the changing environment, MR-1 enhanced chromate resistance by strengthening the function of the cell membrane through genetic mutations and continuously improved its electron transport capacity to reduce Cr (VI) in the acclimation process. The main detoxification pathway of Cr(VI) in MR-1 is the reduction of highly toxic Cr(VI) to less toxic Cr(III), which decreases the damage level to cells. However, the mutated genes in population D40Cr were mainly related to protein secretion and transporter activities (Fig. S4A). It seems that MR-1 can achieve the detoxification of about 100 mg/L Cr(VI) by secreting more proteins involved in metal reduction after the 40-days acclimation. As the acclimation process continued, MR-1 was able to tolerate Cr(VI) of 140 mg/L. As shown in Fig. S4B, mutations in population D80Cr were concentrated on the phosphorelay signal transduction system, the peptidoglycan biosynthesis system and transport. Additionally, adenosine triphosphate (ATP) binding accounted for a large proportion of the molecular function. This result indicated that population D80Cr was adapted to 140 mg/L Cr(VI) owing to its improved metabolic capacity. When bacteria live in a harsh environment, they generate a set of survival methods as part of their long-term evolution process. Among these, the phosphotransferase system plays a crucial role in the utilization of material energy, carbohydrate transport and metabolic system (Deutscher et al., 2006).

3.3 Relationship between nonsynonymous substitutions and Cr adaptation

Nonsynonymous substitution is a nucleotide substitution that changes the encoded amino acid in a manner that is advantageous, neutral or deleterious, while a synonymous

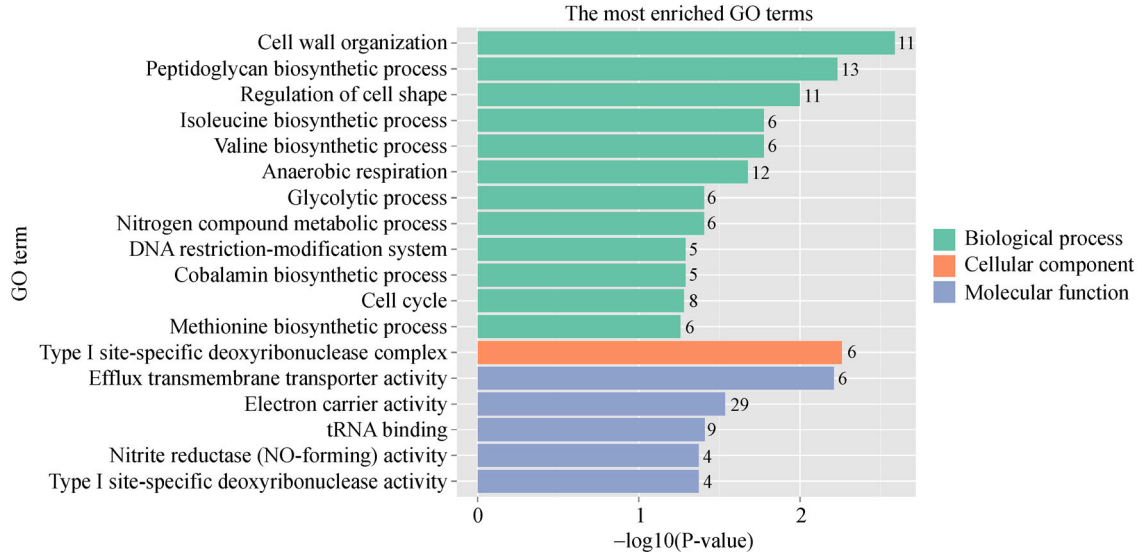

Fig. 3 Most enriched Gene Ontology (GO) terms of mutated genes in population D120Cr. The P-value from the result of enrichment analysis was the X-axes and Y-axes was the GO terms in DAVID functional analysis, which included three categories, namely, biological process, cellular component and molecular function.

mutation is a nucleotide substitution that does not change the encoded amino acid, i.e., it is neutral. Generally, nonsynonymous substitutions and strain fitness are positively associated (Bhatter et al., 2012). Thus, the analysis of nonsynonymous mutations is more helpful for us to understand the factors essential to the improvement in Cr(VI) resistance (Yang and Bielawski, 2000). We calculated the mutation type of all populations in protein-coding DNA sequences. If the ratio of the number of nonsynonymous substitutions (dN) to that of synonymous substitutions (dS) was more than 1.0, then the dN/dS value represented the situation in which the mutations are more beneficial for adaptation (Yang and Bielawski, 2000). The dN/dS ratios for populations D40Cr, D80Cr and D120Cr were 1.08, 1.72 and 2.21, respectively, which indicated that Cr(VI) resistance enhancement was positively correlated with nonsynonymous mutation numbers.

The number of nonsynonymous mutations represents a change in the ability of microorganisms to adapt to a certain environmental condition and is also a stress response to a change in the external environment. We determined the distribution of all nonsynonymous substitutions across the genome circle of MR-1 (Fig. 4(a)), but only a few points of common mutations were shared by different populations, indicating the implementation of different strategies of MR-1 in response to different concentrations of Cr(VI). The largest number of gene mutations are apparent on the outermost circle, and variations in the same locus occurred at different evolutionary times.

Nonsynonymous mutations mainly include missense mutations and stop-gain mutations. The functional effect of stop-gain on a gene was annotated by Snpeff V4.3 (Cingolani et al., 2012). Since a stop-gain mutation terminates the gene expression and affects the protein structure and even the production extremely, there were only 1 to 3 stop-gain mutations occurred in each replicate of the three evolved populations, and these stop-gain mutations either have no fatal effect on bacteria or help bacteria to tolerate high concentrations of Cr.

Figure 4(b) showed 62, 31 and 14 nonsynonymous substitutions occurring in populations D120Cr, D80Cr, and D40Cr, respectively. The number of nonsynonymous variations positively correlated with culture time and Cr (VI) concentration, i.e., the stronger the external stress, the more frequent adaptive mutations occurred. However, MR-1 grown in normal LB broth, i.e., populations D40, D80, and D120, had a very low probability of nonsynonymous mutations, occurring only in 2 sites in population 3 and population 1. The 4 mutated genes are *gltX*, SO_0719, *tldD* and *macB*, which are involved in zinc ion binding and ATP binding and are also the result of evolutionary selection in the natural environment. The results indicated that the increase of nonsynonymous substitutions was an important factor for MR-1 to adapt to high concentration of Cr(VI).

3.4 Function-related mutations for Cr resistance

Missense mutation, a type of nonsynonymous mutation, was the most important reason for the enhanced adaptability and Cr(VI) resistance of MR-1. Functional annotation analysis for missense mutations (Fig. 5) was performed on the DAVID website. Two common func-

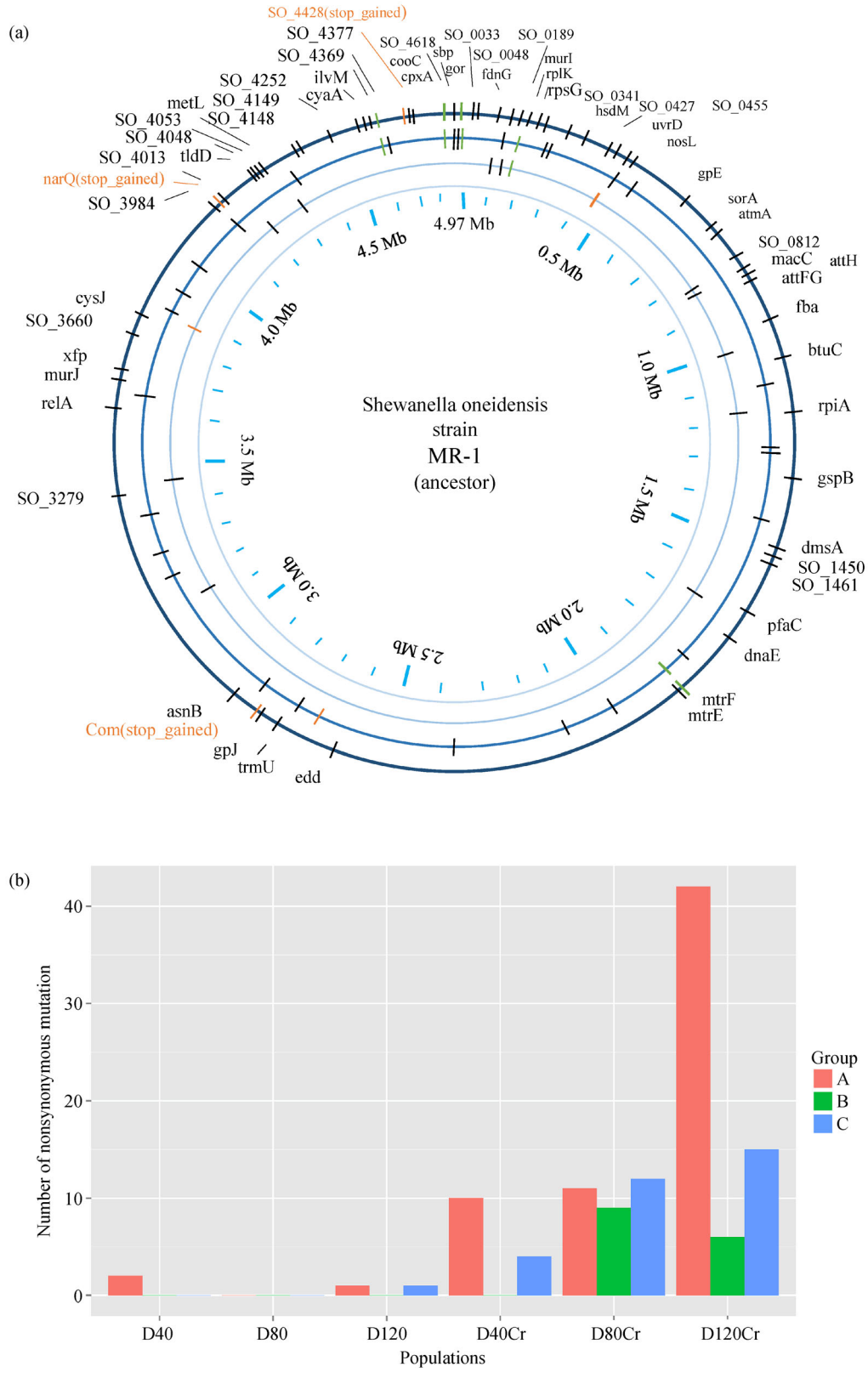

Fig. 4 (a) Distribution of nonsynonymous substitutions across the whole genome between different evolved mutants. Rings from outermost to innermost represent the population D120Cr, D80Cr, D40Cr and ancestral D0, and all genes with nonsynonymous mutations are labeled in coding regions. The orange and aqua dots represent stop-gain mutations and the same mutations that occurred in the evolved populations, respectively. (b) Number of non-synonymous substitution in the 6 evolved populations. Each population has three replicates. The red, green and blue bars represent isolates A, B and C, respectively.

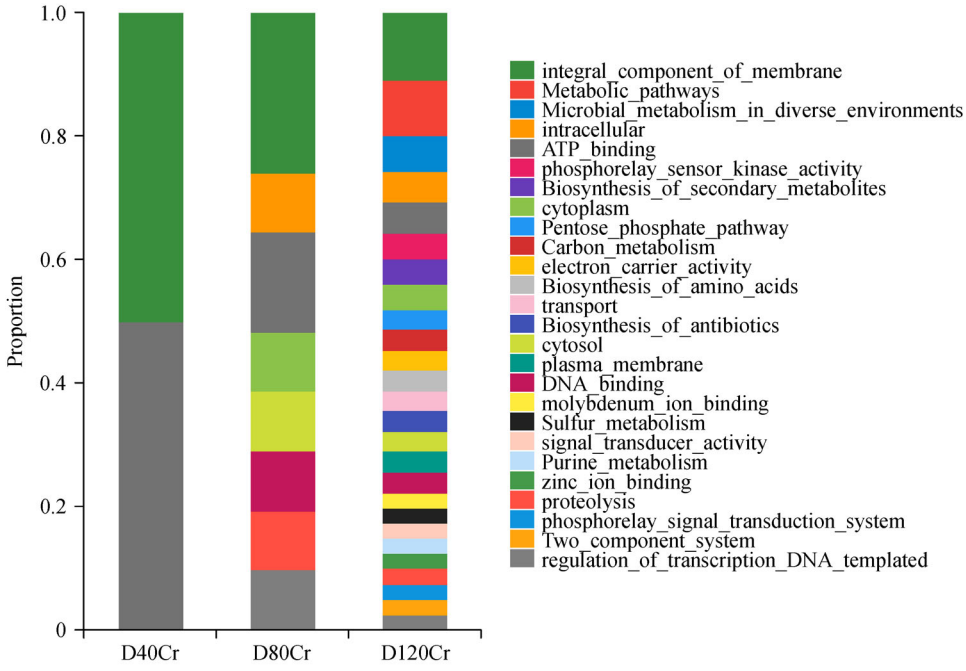

Fig. 5 Functional groups of the missense mutations among populations D40Cr, D80Cr and D120Cr which were inoculated in Cr(VI)-containing medium for 40, 80 and 120 days. Displayed are the different functional categories of the missense mutated genes that use KEGG and GO terms performed with DAVID v.6.8. The percentage value corresponds to the number of missense mutated genes in each functional category.

tional groups, mainly concerned with an integral component of the cell membrane and ATP binding, were shared between populations D40Cr, D80Cr and D120Cr.

The integral component of cell membrane contained the largest number of mutations, and the detailed information on the related genes are listed in Table S7. Several of these genes relate to the efflux pump permease component which is vital to reduce toxic substances intracellularly for detoxification, and the other genes are involved in ABC-type export system and transporter activities for sugars, ions and amino acids (Puentes-Téllez et al., 2013) and a secretion system of the inner membrane protein for maintaining the normal function of the cell. Mikolay et al. revealed that the ABC-transporter *AtmA* protected *C. metallidurans* and *E. coli* against cobalt and nickel toxicity (Mikolay and Nies, 2009). The missense mutation of *atmA* in MR-1 could be beneficial for enhanced Cr resistance. The inner membrane platform protein *gspL* takes part in energy conversion (Korotkov et al., 2012) and plays a crucial role in the cytoplasmic secretion of ATPase. The gene *macC* encodes a kind of *c*-type cytochrome that contributes to U(VI) reduction (Holmes et al., 2009), and the mutation on this gene may improve Cr reduction of MR-1 cells. Being exposed to acute Cr, transport and binding protein SO_3279 was significantly expressed (Torres-García et al., 2011) and the mutation on SO_3279 was directed at the increasing chromate in long-term conditions. In addition, membrane-associated proteins and transporters, including SO_4053 as the most direct protection barrier, are crucial to counter different types of environmental pressure, such as cold shock (Gao et al., 2006). SO_4148 is a toxin secretion-related gene involved in ultraviolet A irradiation response (Qiu et al., 2005); and the missense variation in this gene occurred to avoid the accumulation of toxins. Notably, the gene *mexF* in population D80Cr has been identified with multidrug resistance and is required for resistance to antibiotics (Groh et al., 2007), which may be also important for Cr resistance.

The second common functional group with missense mutations to populations D40Cr, D80Cr and D120Cr was involved in ATP binding since the basal metabolism of cells and the rapid reduction of Cr(VI) require plenty of energy. Therefore, we measured the ATP content of populations D40Cr, D80Cr and D120Cr at the 24th h and 48th h. Figure S5 showed that the average ATP concentration in cells from populations D40Cr, D80Cr and D120Cr were 500–1000 fold higher than that of the ancestral D0 population. The result also confirmed that the mutation of related genes are reflected by the ATP content, which enhanced the metabolic activity and detoxification capacity of MR-1 exposed to Cr(VI). In population D120Cr, the mutated genes related mainly to metabolic pathways (*edd*, *xfp*, *dnaE*, *ilvM*, *asnB*, *metL*, *fba*, *murI*, *fdnG*, *rpiA*, and *cysJ*). Among these, there are 7 genes that are related to microbial metabolism in diverse environments (*edd*, *xfp*, *metL*, *fba*, *fdnG*, *rpiA* and *cysJ*), electron carrier activity (SO_4048, *dmsA*, *mtrF* and *fdnG*), plasma

membrane (*btuC*, *attFG*, *relA* and SO_0455), DNA binding (*uvrD*, *dnaE*, SO_4428 and *hsdM*) and signal transduction (SO_0427, SO_4053 and SO_0341). In addition, the missense mutation in *gor* may also play a vital role in enhancing the resistance of MR-1 to Cr(VI) as the gene codes for glutathione-disulfide reductase to maintain a cytoplasmic redox homeostasis.

3.5 Proposed adaptation mechanism of *S. oneidensis* MR-1 to Cr(VI)

During the 120-day acclimation of *S. oneidensis* MR-1, the bacterium was able to adapt rapidly to conditions with increasing concentration of Cr(VI). Ultimately, we obtained evolved MR-1 mutants which had improved resistance to Cr. While populations D40, D80, and D120 cultured in normal LB broth had no change in Cr(VI) resistance and reduction capacity, the population D120Cr, which is the ancestral D0 inoculated in Cr(VI)-containing LB broth for 120 days, can tolerate Cr(VI) up to 190 mg/L. Although the amount of reduced Cr(VI) in populations D40Cr, D80Cr, and D120Cr are limited to about 90, 96 and 81 mg/L due to nutrient limitation, D120Cr can reduce Cr (VI) of 160 mg/L with doubled nutrient levels in the medium. These evolved mutants have evolved a fast reducing ability for Cr(VI). Thus, the acclimation of microorganisms is a viable and effective method for enhancing the transformation of pollutants in microbial technologies.

Through mutation analysis, we illustrated the adaption process of MR-1 in the subculture of Cr(VI) medium and the mechanism of enhanced resistance to Cr (Fig. 6). As the toxic Cr(VI) molecules enter intracellularly through the sulfate uptake channel (A in Fig. 6), MR-1 needs to continuously strengthen its detoxification process. One of the important strategies is to reinforce the integral membrane components including efflux pump components (SO_3492, SO_4377, and SO_3279) that can transfer intracellular Cr to the cell exterior rapidly, thus decreasing the accumulation of toxic substances. ABC transporters (*atmA*, *btuC*, *attFG*, and *macC*) transport sugars, ions and amino acids and are also play important roles in heavy metal resistance (Puentes-Téllez et al., 2013). Another major feature focuses on ATP binding which provides sufficient energy for reductase and transporter activity. Additionally, rapid signal transmission (e.g., SO_0427, SO_4053 and SO_0341) can ensure the cell response to the changing environment in a timely manner. Enhanced electron transfer by activating the activity of the electron carrier function (SO_4048, *dmsA*, *mtrF*, and *fdnG*) may be also an indispensable step in the rapid reduction of Cr(VI). Since intracellular Cr(III) can disrupt proteins and DNA by forming adducts, mutations on genes involved in DNA binding and regulation may enhance the ability to repair such damages. Notably, reactive oxygen species are generated during the reduction process of heavy metals (Choi and Hu, 2008), and the missense mutation of *gor* may play a significant role in strengthening the detoxification of Cr(VI).

4 Conclusions

In summary, there is a positive relationship between

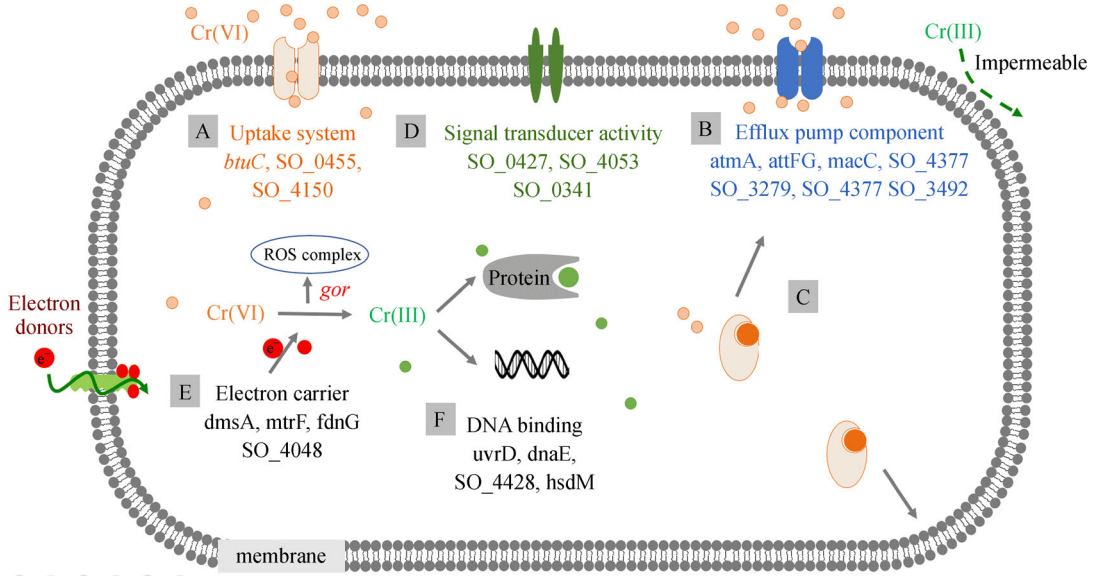

Fig. 6 Missense mutated genes across integral component of the membrane in *Shewanella oneidensis* MR-1. (A) Sulfate uptake channel which is also used by chromate. (B) Efflux pump component of membrane. (C) Transporter encoded by genes which transport substances to ensure the metabolic function. (D) Signal transducer activities. (E) Electron carrier enzyme to take part in the reduction of Cr(VI). (F) DNA binding.

mutated gene number and the adaptation of *S. oneidensis* MR-1 in LB broth containing increasing Cr(VI). The enhanced resistance and reduction capacity to Cr(VI) may be primarily ascribed to gene mutations related to the membrane efflux components, the regulation of transporter activity and the electron carrier activity. This investigation looked at the whole genome level and has provided a comprehensive illustration of how *S. oneidensis* MR-1 respond to Cr pollutant during long-term acclimation, showing potential for future environmental engineering application.

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