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Functional genomic analysis of *Bacillus cereus* BC4 strain for chromium remediation in contaminated soil

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ABSTRACT

Soil provides a habitat for microorganisms that can mitigate metal contamination. This study presents *Bacillus cereus* BC4 strain, which shows significant potential for metal pollution remediation. This bacterium achieved a 98.6 % reduction in Chromium (VI) concentrations from 300 mg/L to negligible levels under specific conditions (pH 8, 37 °C, and 120 rpm agitation) in LB medium. The complete genome of *Bacillus cereus* BC4 was sequenced using Oxford Nanopore Technology, revealing a circular chromosome and a plasmid with a total of 5537,675 base pairs and a $G + C$ content of 35.44 %. Fourteen genes critical for Cr metabolism were identified. qRT-PCR demonstrated that under low Cr(VI) stress, two genes, *chrA* and *nitR1*, were up-regulated, indicating their role in Cr resistance. The genome revealed gene clusters essential for resilience against various metals, including chromium, arsenic, copper, manganese, and cadmium, as well as for synthesizing secondary metabolites crucial for survival and adaptation. Additionally, genes associated with biopolymer synthesis were identified, emphasizing the organism's diverse genetic capabilities. This genomic study led to the submission of the complete genome to GenBank (CP101135), enhancing the understanding and potential of *Bacillus cereus* BC4 in chromium remediation and environmental restoration.

Environmental Implication

The *Bacillus cereus* BC4 shows significant promise for environmental rehabilitation, particularly in chromium-contaminated soils. With a remarkable 98.6 % reduction of Cr(VI) levels, this strain highlights the potential of utilizing microbial genomics for bioremediation. By elucidating the genetic mechanisms behind chromium metabolism, including the identification of critical genes, this research paves the way for innovative strategies to remediate heavy metal pollution, promoting healthier ecosystems. The findings can contribute to sustainable agricultural practices and soil health restoration, underscoring the importance of harnessing microbial capabilities for environmental conservation.

1. Introduction

Chromium is recognized as one of the 8th most harmful elements to human health. Chromium (Cr) compounds usually exist in the form of Cr (III) and Cr(VI) in the environment (Thacker et al., 2006). The Cr(VI) is 100 folds toxic than Cr(III). Various microorganisms are capable of confiscating the Cr(VI) in contaminated environments. Some microorganisms, mainly bacteria including *Staphylococcus aureus* (Tariq et al., 2019), purple non-sulfur photooxygen bacteria (Upadhyay et al., 2017), archaea (Singh et al., 2015), *Enterobacter, Thiobacillus* and *Pseudomonas* 8 (Zhou et al., 2016; Kumari et al., 2015; Kang et al., 2017), sulfate-reducing bacteria and many other different species, genus, can reduce Cr(VI) to Cr(III). Some fungi such as yeast (Fernandez et al., 2017) and mold (Gu et al., 2014) have also been reported for remediation of Cr(VI)-containing wastewater.

Abbreviations: Cr, Chromium; KEGG, Kyoto Encyclopedia of Genes and Genomes; COG, Cluster of Orthologous Groups; GO, Gene Ontology; qRT-PCR, quantitative real-time PCR; ONT, Oxford Nanopore Technologies; Nfs, Nitrate reductase.

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Methods based on biotransformation and bioremediation often use the catabolic activities of microorganisms themselves to degrade, transform, or absorb large amounts of toxic substances (Sharma et al., 2021). The microorganisms use different mechanisms to counteract heavy metal toxicity, for example adsorption, oxidation, methylation, and reduction of heavy metals to non-toxic forms (Hossan et al., 2020). The tolerance of bacteria against high concentrations of metals is an important property of microorganisms. Microbial conversion of Cr(VI) is an enzymatic catalytic reaction, which is attributed to membrane-bound enzymes and soluble reductases (Pradhan et al., 2016). In addition, the reduction of Cr(VI) to Cr(III) has also been revealed to be effectively accomplished by direct electron transfer through bacterial hairs. Chromate ions can also be transferred to the intracellular space via sulfate transport channels, while nicotinamide adenine dinucleotide phosphate (NADPH) can be involved in Cr(VI) reduction via electron transfer (Ghosh et al., 2021).

In the recent years, multi-omics approaches, such as genomics, transcriptomics, proteomics and metabolomics, have been rapidly got advancements in the microbial remediation of heavy metal contamination (Pirih and Kunej, 2017). Genomics and transcriptomics using important biochemical reaction pathways have paved the way to study the underlying mechanisms for microbial remediation of metal contaminated environments. Previous studies have demonstrated that there are many genes related to Cr(VI) tolerance and removal in Cr-contaminated soils. The *Escherichia coli* FACU was a Cr(VI) reductase that could generate ChrR protein (Mohamed et al., 2020). The genome of *Serratia* sp. S2 had Cr(VI)-reducing gene proteins, for example, nitroreductase (RutE, NfsA, NfsB, and Ydja), NAD(P)H-dependent azoreductase (AcpD), and flavoprotein reductase (Ribf, ChrT and SsuE) (Dong et al., 2018). Genomic analysis of *Bacillus cereus* NWUAB01 showed that the ChrA protein was involved in chromate reduction and transport (Ayangbenro and Babalola, 2020). A total of 361 proteins have been up-regulated under Cr(VI) stress, including enzymes of relevant to uptake, intracellular reduction, DNA repair and Cr(VI) efflux, ROS detoxification, as well as electron transfer mediating extracellular reduction, Quorum sensing and chemotaxis-related proteins (Chai et al., 2019).

The *Bacillus cereus* is a complex group, which belongs to the genus Bacillus and is ubiquitously present in natural environments. This species consists of many gram-positive, spore-forming, facultative anaerobic lineages that are closely linked to one another (Ehling-Schulz et al., 2019; Messelhäußer and Ehling-Schulz, 2018; Rasko et al., 2005; Stenfors Arnesen et al., 2008). However, current research frequently relies on conventional methods that fall short in thoroughly unraveling the mechanisms at play. Moreover, only a handful of studies have delved into the biological role and mechanisms of the *Bacillus cereus* at the genetic level, leaving a significant gap in our understanding.

In this study, we isolated a *Bacillus cereus* strain, designated as *Bacillus cereus* BC4, from soil contaminated with hexavalent chromium in our laboratory. We optimized the conditions for reducing Cr(VI) by *Bacillus cereus* BC4 and sequenced the entire genome of the strain BC4 using Oxford Nanopore Sequencing technology. Additionally, we acquired comprehensive genomic and proteomic data. This research enhances our understanding of this bacterium and lays a theoretical foundation for its application in the remediation of heavy metal pollution.

2. Materials and methods

2.1. Strains filtration

Soil samples were collected from a rice paddy soil located in Wuhan, China. The weight of 10 g soil was added to 100 mL of sterile water and thoroughly mixed. Then, 1 ml was taken to 5 mL of Luria-Bertani (LB) liquid medium for 3 days. The resultant product of bacterial liquid was then transferred to 1 mL of LB medium with a Cr(VI) concentration of 100 mg/L (Tan et al., 2023). The organisms were cultured in liquid LB medium of various metal concentrations ranging from 100 to 500 mg/L. The plates were kept at 37 °C for 48 h for microbial growth. The method of "Water Quality - Determination of Hexavalent Chromium Diphenylcarbazide Spectrophotometry" was used to calculate the content of Cr (VI) (GB7467–1987). The bacterial liquid containing Cr(VI) was sampled every 12 h, and then the diphenylcarbazide color developer was added. After 10 min of color reaction, the absorbance was measured at 540 nm with a spectrophotometer, and the reduction rate of Cr(VI) was computed.

2.2. Optimization of Cr(VI) environmental parameters

To identify the optimal conditions for reducing Cr(VI) by the *Bacillus cereus* BC4 strain in LB medium, we systematically assessed various environmental factors. These included pH levels (ranging from 4.0 to 10.0), temperature settings (25 °C, 30 °C, 37 °C, and 40 °C), aeration rates (100, 120, and 150 rpm), and initial Cr(VI) concentrations (spanning from 100 mg/L to 1500 mg/L). Each set of experiments was conducted in triplicate to guarantee the reliability of our findings.

2.3. Whole genome sequencing

Bacillus cereus strain BC4 was isolated in our laboratory and stored at −80 °C. The strain was activated, cultured overnight in LB liquid medium at 37 °C while shaking at 120 rpm, and culture was then harvested. To create a 1D library, the high-quality DNA was extracted using a Qiagen kit. The DNA was then sequenced using the Oxford Nanopore Technology sequencer PromethION to collect the raw sequencing data (Brown et al., 2023; Siregar et al., 2021; Delandre et al., 2024). The following four methods were used to detect whether the DNA is qualified: (1) Whether the appearance of the sample contains foreign matter; (2) 0.75 % agarose electrophoresis: to detect whether the sample was degraded and the size of DNA fragments; (3) Nanodrop: to detect DNA purity (OD260 /280 was between 1.8–2.0; OD 260/230 was between 2.0–2.2) (4) Qubit: precise quantification of DNA. After confirmation of DNA quality, the BluePippin automatic nucleic acid gel cutter was utilized to recover DNA with a specific fragment size, followed by DNA gel cutting subjected to damage repair and end repair. NBD114 and NBD103 kits were used to connect the DNA ends. Barcode tags, magnetic beads purification, ligation of the sequencing adapters included in the kit, and finally quantitative detection of the constructed DNA library with Qubit. After establishing the library, a definite volume and concentration of DNA library was added to the Flow cell and transferred to the PromethION sequencer for real-time single-molecule sequencing.

2.4. Whole genome data assembly

After confirming the quality, the 2nd and 3rd generation data were mixed and assembled with unicycler, then corrected with pilon joined with the 2nd-generation sequencing data (Mastriani et al., 2022; Bonfiglio et al., 2024). The corrected genome was tested for loop formation using its own script. After excising the redundant parts, the final genome sequence was obtained by moving the origin of the sequence to the replication start site of the genome using circulator (https://github.com/sanger-pathogens/circlator) for the sequences that have become loops.

2.5. Function annotations

After obtaining the complete genome, the automated annotation pipeline of RAST (http://rast.nmpdr.org) was applied to predict and annotate the genome structure (Valle et al., 2019; Barnett et al., 2001). To predict the function of *Bacillus cereus* BC4 proteins, we used BLASTP to compare the amino acid sequences of all proteins with the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Galperin et al., 2015),

Cluster of Orthologous Groups of proteins (COG), Swiss Prot (http://www.expasy.ch/sprot), Gene Ontology(GO) (Lagesen et al., 2007) databases were compared to obtain the corresponding functional annotation information. Since sequence alignment would result in multiple alignment results, to ensure accuracy, we retained the optimal alignment result as the gene annotation information. In addition, we used RNAmmer (Lowe and Eddy, 1997) and tRNAscan-SE (Zhang et al., 2000) to predict rRNA genes and tRNA genes in the genome, and IS Finder (Benson, 1999) to predict insertion sequences in the genome.

After the completion of structural and functional annotations, the results were combined and uploaded to NCBI. At the same time, the nuclear genome sequencing depth, GC distribution, GC-skew, and genome structure were analyzed with its own script, and the nuclear genome circle map was drawn with circus (Krzywinski et al., 2009).

2.6. Genes related to chromium and other metal metabolism

To determine the appropriate functional annotation, the amino acid sequences of the genes were equated to the databases for the Kyoto Encyclopedia of Genes and Genomes (KEGG), Cluster of Orthologous Groups (COG) and Gene Ontology (GO). For gene annotation, the best comparative findings were considered.

2.7. Relative fluorescence quantitative PCR

To explore the altered expression of genes related to hexavalent chromium efflux and reduction under Cr(VI) stress, we employed relative fluorescence quantitative PCR (Li et al., 2021; Man et al., 2023). The *Bacillus cereus* BC4 was cultured in LB medium until it reached mid-exponential growth phase. Subsequently, 2 % of this inoculum was introduced into LB media with Cr(VI) concentrations of 0, 100, and 300 mg/L, respectively. Cultures were then incubated under chromium stress at 37 °C and 120 rpm for 24 h. After incubation, cells were harvested by centrifugation for 5 min at 8000 rpm and 4 °C, followed by two washes with phosphate-buffered saline (PBS) at pH 7.4. The samples were sent to Wuhan Bioyigene Biotechnology Co., Ltd., for quantitative real-time PCR (qRT-PCR) analysis. The qRT-PCR was conducted using the Applied Biosystems StepOnePlus™ Real-Time PCR System, with the following protocol: an initial cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The specificity of the primers was verified before conducting the formal experiments. The primers used in the qRT-PCR are detailed in Table S4. The 16S rRNA gene was used as a normalization control. Data from qRT-PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method, allowing for precise quantification of gene expression alterations under stress conditions.

3. Results

3.1. Strains filtration

Six strains exhibiting Cr(VI) removal capabilities were isolated from soil samples and initially labeled as Cr-1, Cr-2, Cr-3, Cr-4, Cr-5, and Cr-6. These strains were cultured in LB medium containing 100 mg/L of Cr (VI) at 30 °C and 150 rpm for a duration of 3 days, with their Cr(VI) removal efficiencies presented in Table S1. The results demonstrated that all six strains were capable of removing Cr(VI) to some extent. Notably, strains Cr-2, Cr-4, Cr-5, and Cr-6 exhibited superior removal efficiencies, achieving Cr(VI) removal rates exceeding 70 % after the 3-day treatment period. Among these, the Cr-4 strain distinguished itself by achieving the highest Cr(VI) removal rate of 99.28 %, thus, making it the primary focus for further study under the designation *Bacillus cereus* BC4. Gram staining of this strain revealed it to be a Gram-positive rod.

3.2. Influence of different factors on the reduction of Cr(VI)

In the context of Cr(VI) remediation, selecting the optimal environmental parameters is crucial for the efficacy of microbial treatment processes. Consequently, assessing the impact of various environmental factors on the Cr(VI) removal efficiency is of paramount importance.

3.2.1. pH

Fig. 1(a) shows the significant effects of pH on the removal efficiency of hexavalent chromium by the strain BC4. At pH 5.0, the removal of Cr (VI) was only 65.6 %, which may be due to the lower pH inhibiting the growth and metabolic activities of the bacteria (Tomasz et al., 2024). However, the removal rate increased with increasing pH, and peaked at pH 8.0 with 99.09 % removal after 72 h. This suggests that the reductase activity of *Bacillus cereus* BC4 may be higher under alkaline conditions or the cell membrane may be better permeable, which favors the uptake and reduction of Cr(VI) (Mao et al., 2023). There was no significant change in the removal rate at pH values above 8.0, which may be because the reduction capacity of the bacteria is already maximized in this pH range. This trend suggests that an overly acidic environment may inhibit bacterial growth and metabolism due to reduced bacterial biomass and diminished reductase activity, consequently affecting the strain's capacity for Cr(VI) removal. Despite these challenges, the strain BC4 demonstrated the capability to remove over 70 % of Cr(VI) across a broad pH spectrum, indicating its adaptability. Nonetheless, the data suggests that the strain operates more efficiently in alkaline conditions. Acidic conditions might also negatively impact protease activity and the structure of certain proteins, further inhibiting the bioreduction potential of Cr(VI).

3.2.2. Temperature

Fig. 1(b) demonstrates that the Cr(VI) removal rate by the strain BC4 is optimally high within the temperature range of 37–40 °C, peaking at a remarkable 97.5 % efficiency at 37 °C. This coincides with the optimum growth temperature of *Bacillus cereus* BC4, indicating that the metabolic activity and reductase activity of the bacterium are strongest at this temperature. Conversely, at a lower temperature of 25 °C, the removal rate significantly drops, likely due to the reduced enzyme activity that colder conditions induce, which in turn diminishes the strain's capacity for Cr(VI) removal. Low temperatures adversely affect the growth of bacterial strains by decreasing cell membrane fluidity, which hampers the cell's nutrient uptake system and ultimately inhibits growth. On the other hand, high temperatures can alter membrane structures and deactivate key mechanisms such as reductase activity or protein synthesis. Consequently, the ideal temperature for Cr(VI) remediation using the strain BC4 is pinpointed at 37 °C.

3.2.3. Ventilation

Fig. 1(c) illustrates the pivotal role that shaker speed plays in Cr(VI) removal efficiency by the strain BC4. At a modest speed of 100 rpm, the Cr(VI) removal effectiveness was limited. However, a notable increase in efficiency occurred as the speed was adjusted to 120 rpm, enabling near-complete Cr(VI) removal within a span of 24 h. Pushing the speed further to 150 rpm led to a decrease in the Cr(VI) removal rate, suggesting an optimal shaker speed of 120 rpm for maximal efficiency in Cr (VI) removal by the strain BC4.

3.2.4. Effect of initial Cr(VI) concentration

Fig. 1(d) highlights that Cr(VI) removal is remarkably efficient at a concentration of 100 mg/L, achieving an impressive removal rate of 99.8 %. However, as the concentration of Cr(VI) increases, a gradual decrease in the removal rate is observed. Specifically, at a concentration of 300 mg/L, the removal rate slightly drops to 98.6 %, and a more significant reduction is noted at 500 mg/L, where the rate plummets to 22.8 %. At an elevated concentration of 1500 mg/L, Cr(VI) removal becomes negligible. This pattern suggests that higher concentrations of Cr(VI) exert a toxic effect on the bacteria, inhibiting their growth and, consequently, impacting their ability to remove Cr(VI).

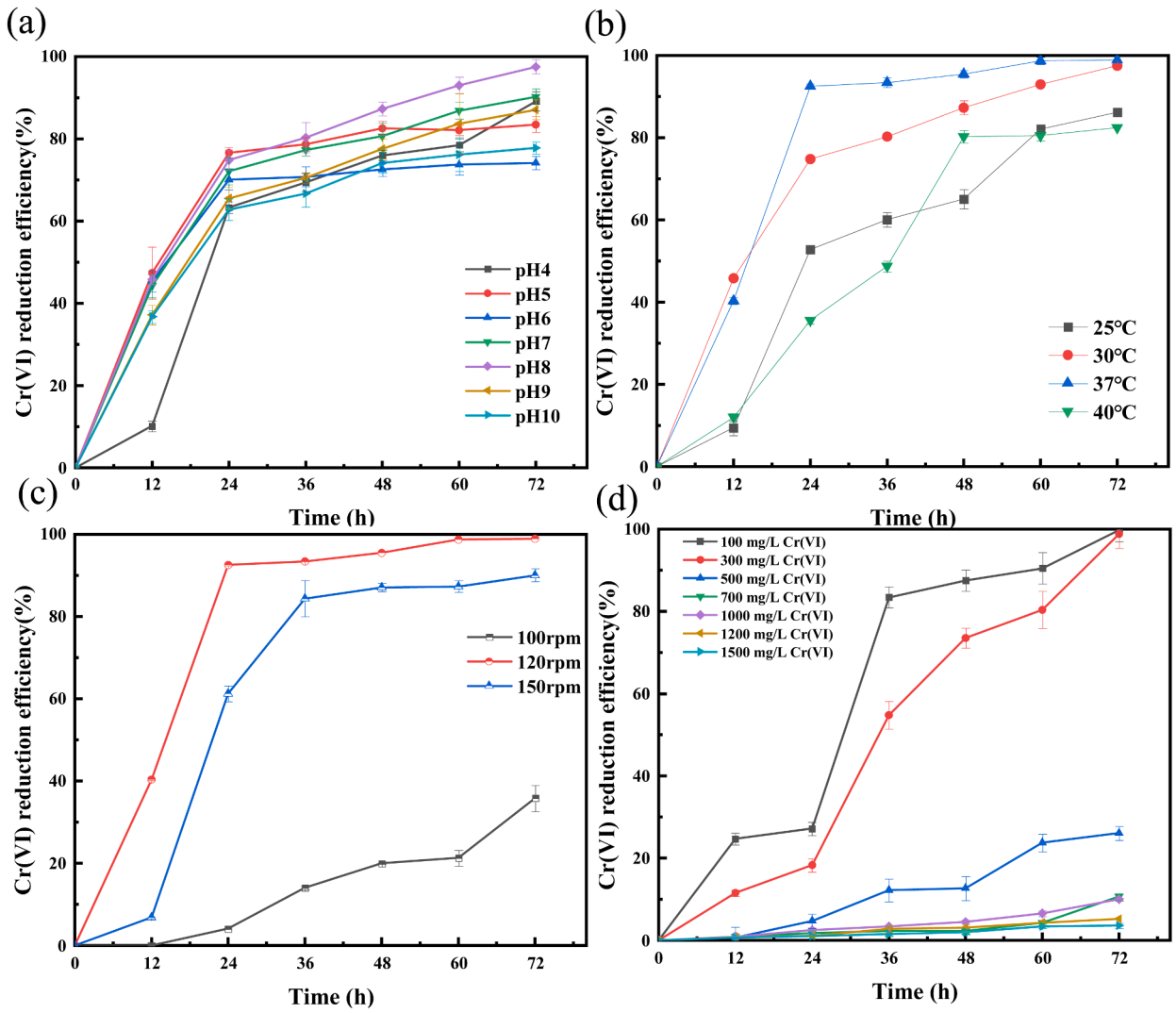

Fig. 1. Effect of different parameters on Cr(VI) reduction of *Bacillus cereus* BC4. **a** Effect of pH. **b** Effect of temperature. **c** Effect of ventilation. **d** Effect of initial. Cr (VI) concentration. The data are presented as the mean (n = 3)±standard deviation (S.D).

3.3. Genome assembly and basic characteristics

The experiment was carried out using the Canu v1.5 software to build filtered subreads in accordance with the standard approach given by Oxford Nanopore Technologies (ONT) (Koren et al., 2017). The assembled genomes were then further corrected using Pilon software (https://github.com/broadinstitute/pilon) and second generation data (Walker et al., 2014). The genomic features of *Bacillus cereus* BC4 were shown in Table 1, yielding a total genome size of 5537,675 bp. Based on the assembly results, it is achieved that the entire genome of *Bacillus cereus* BC4 is made up of two contigs with a combined GC content of 35.44 %, including a circular chromosome with a length of 5245,328 bp and a circular plasmid with a length of 292,347 base pairs, both of which have respective GC contents of 35.55 % and 33.50 %, respectively. In addition to 253 plasmid genes and 5335 projected chromosomal genes, there were 106 tRNAs, 39 rRNAs and 267 sRNAs. CDS (coding sequence) is a segment of sequence on the genome that encodes a protein with a start codon at the 5′ end and a stop codon at the 3′ end. Based on the prediction of the coding region by this feature, a total of 5563 complete CDSs were obtained, most gene fragments are <1000 bp, and the distribution of their encoded protein lengths is shown in Fig. 2.

Table 1
Bacillus cereus BC4 genome features.

Attribute	Values
Genome size (bp)	5537,675
GC content (%)	35.44
Total genes	238,417
CDS (protein)	5563
tRNA genes	106
rRNA genes	39
ncRNA genes	267

According to the genome sequence of *Bacillus cereus* BC4, its assembly was at the level of a completed map. The chromosome circle map and plasmid circle map were drawn by Circos software to display the distribution of genes, annotation information, GC content and other elements in the genome of *Bacillus cereus* BC4 strain. In this case, the chromosome circle diagram and plasmid circle diagram were shown in Fig. 3. From outside to inside were coding gene (sense strand), coding gene (negative-sense strand), tRNA (orange) and rRNA (purple), CRISPR, prophage and gene island, GC ratio, GC-skew, and sequencing depth. The nuclear genome circle map showed that the bacterial genome covered most of the COG functional annotation types, indicating a high degree of genome integrity.

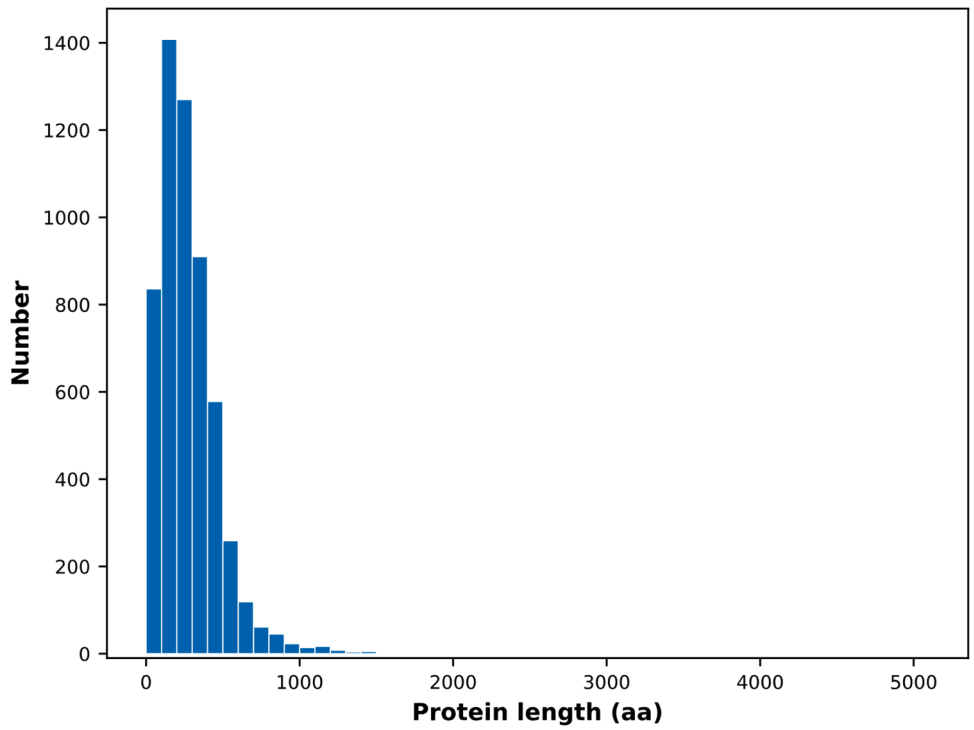

Fig. 2. Gene length distribution of *Bacillus cereus* BC4.

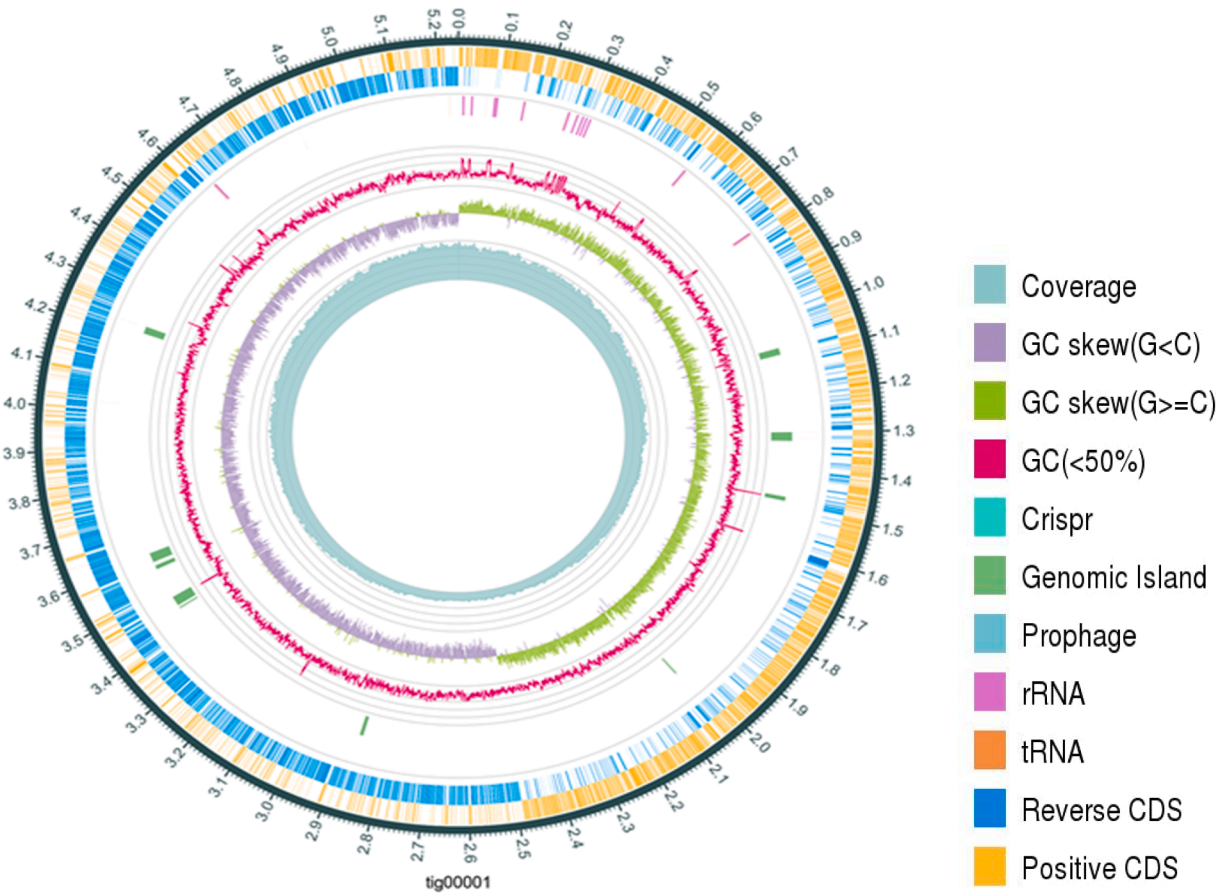

Fig. 3. Nuclear genome circle diagram of *Bacillus cereus* BC4.

3.4. Plasmid analysis

Plasmids are self-replicating DNA molecules that are ubiquitous in prokaryotic cells and independent of nuclear DNA. The analysis of the plasmids in the assembly results showed that tig00002 may be a plasmid sequence. The detailed results are shown in Table 2.

3.5. Genome annotation and gene functional analysis

The whole genome sequence of *Bacillus cereus* BC4 was predicted by Glimmer (Delcher et al., 2007), and there were 5563 genes, including 5335 chromosomal genes and 253 plasmid genes. The gene activities were determined using BLAST software's annotation of the genes against the database, which could clarify the biological characteristics and functions of *Bacillus cereus* BC4 at the molecular level and gave a theoretical foundation for the creation and use of the strain.

3.6. GO function analysis

The annotation of the GO database comes from is achieved from the Quick GO database in the Interpro database. Therefore, the database results contain two types of annotation information: Interpro database annotation information and Quick GO database annotation information. Because three types of GO database overlap each other, that is, a gene can be annotated by multiple branches of GO at the same time, there will be gene duplication annotations on the result. Therefore, for genes which are annotated with multiple GO categories at the same time, their functions are further determined by the information between different categories. To gather information on GO annotations, the protein sequences encoded by CDS were matched with the GO database, and the analysis of GO functional grouping was carried out. (Fig. 4). The three major ontologies of GO are represented by different colors, with red representing biological processes, purple representing cellular components and green representing molecular functions (Li et al., 2010). The genes predicted from the *Bacillus cereus* BC4 genome were classified into 30 majors in functional grouping.

Among the molecular function annotations, the number of genes with catalytically active molecular functions was the most being 1777, accounting for 48.68 % of all molecular function annotation genes; followed by genes with binding functions, with 1231 genes, accounting for 33.73 %. The genes annotated by cellular components were mainly concentrated in cells, cell membranes, and both components. It can be seen from Fig. 4 that the number of genes related to cell, binding, catalysis, metabolism and other functions was relatively large, indicated that strain *Bacillus cereus* BC4 had higher activities in differentiation, enzyme catalysis and metabolism.

3.7. COG functional analysis

The development of bacterial, algal, and eukaryotic species is the basis of the COG database. The assembled single gene of *Bacillus cereus* BC4 was examined in the COG database of BLAST (Raymond et al., 2010). The COG database can be divided into twenty-five categories according to their functions. The protein sequences can be annotated into specific COG functional categories through alignment. Orthologous sequences make up each COG cluster, allowing the purpose of each sequence to be deduced. The predicted CDS sequence of this strain was compared with the COG database using Glimmer 3.02 and BLAST software to determine the annotation of coding genes. We were able to identify coding genes with unknown roles as well as coding genes involved in basic metabolism, cell biological processes, signal transduction, information storage, processing. The successful annotation of 4745 coding sequences into COG totaled (Fig. 5).

In addition to universal sequences and unidentified essential sequences, there are 423 amino acid transport and metabolism sequences (8.9 %), 424 transcription sequences (8.9 %), 252 inorganic ion transporter and metabolism sequences (5.3 %), 220 energy production and conversion sequences (4.6 %), 175 sequences related to DNA replication, recombination and repair (3.7 %) and 276 sequences related to cell wall/membrane/envelope biogenesis (5.8 %). Inorganic ion transporter genes, for example, may play a role in regulating bacterial absorb and discharge of Cr (VI). The affirmation of Cr(VI) metabolism-related proteins may be regulated by cell membrane synthesis genes and amino acid transport genes. Bacillus may have genes for DNA replication, recombination, and repair, which suggests that it can self-heal broken DNA to withstand the damaging effects of Cr(VI).

Table 2
Bacillus cereus BC4 Plasmid profile analysis.

Contig	tig00001	tig00002
Length (bp)	5245,328	292,347
Length aligned to database (bp)	983,744	292,345
% contig	18.75	100.00
GC content (%)	35.55	33.50
Topology	Circular	Circular
Sequencing depth (X)	242.87	282.95

Note: Contig: Contig sequence id;Length (bp):length of Contig sequence (bp); Length aligned to database (bp):length of Contig sequence aligned to plasmid database (bp); % contig:percentage of Contig sequence aligned to plasmid (%); GC content (%): GC content of Contig sequences (%); Topology:topology of Contig sequences;Sequencing depth (X):triple sequencing depth of Contig sequences).

3.8. KEGG function analysis

The most central database of KEGG is KEGG PATHWAY, which divides biological metabolic pathways into seven major categories. The KEGG PATHWAY metabolic pathway annotation makes it easy to find all the genes on the annotation related to the exercise of a certain class of function, and also to discover genes that play an up- or down-regulated role in the metabolic pathway. Fig. 6 was obtained after the secondary classification statistics of the KEGG annotation of the *Bacillus cereus* strain BC4 genome. 3614 genes were annotated by the strain genome KEGG and divided into 33 secondary metabolic pathways. Most of them belong to the following categories: 149 signal transduction sequences in environmental information processing (29.6 %), 253 carbohydrate metabolism sequences (17 %), 260 amino acid metabolism sequences (12 %), 220 genetic information processing sequences (8 %),140 energy metabolism sequences (6.5 %), 94 translation sequences (5.9 %) and 5 transcription sequences (5.7 %). Notably, most of the genes involved in membrane transport, material transport and metabolism, and energy metabolism in the Bacillus genome were identified in the annotated results of the KEGG and COG databases.

The damage to bacterial DNA after Cr(VI) entering cells in a chromium-contaminated environment might be related to cell membrane transport, material translocation and metabolism. Membrane transport, energy metabolism, intracellular transport, and enzymatic catalysis are all interconnected processes that may control the secretion and movement of the enzyme Cr(VI) reductase to reduce Cr(VI).

3.9. Cluster analysis of secondary metabolite biosynthesis genes

The secondary metabolites of bacteria and fungi constitute a wealth of potentially medicinally valuable bioactive compounds, and many of them are being used as antibiotics, cholesterol-lowering drugs or antitumor agents. The genes responsible for the synthesis of a particular secondary metabolite are often clustered together at a specific location on the chromosome, a characteristic of genes referred to as "secondary metabolite gene clusters". This genetic structure provides a theoretical basis for detecting secondary metabolite synthesis pathways by simply localizing gene clusters. Using antiSMASH (Weber et al., 2015; Medema et al., 2011), a Hidden Markov Model based on the genetic information

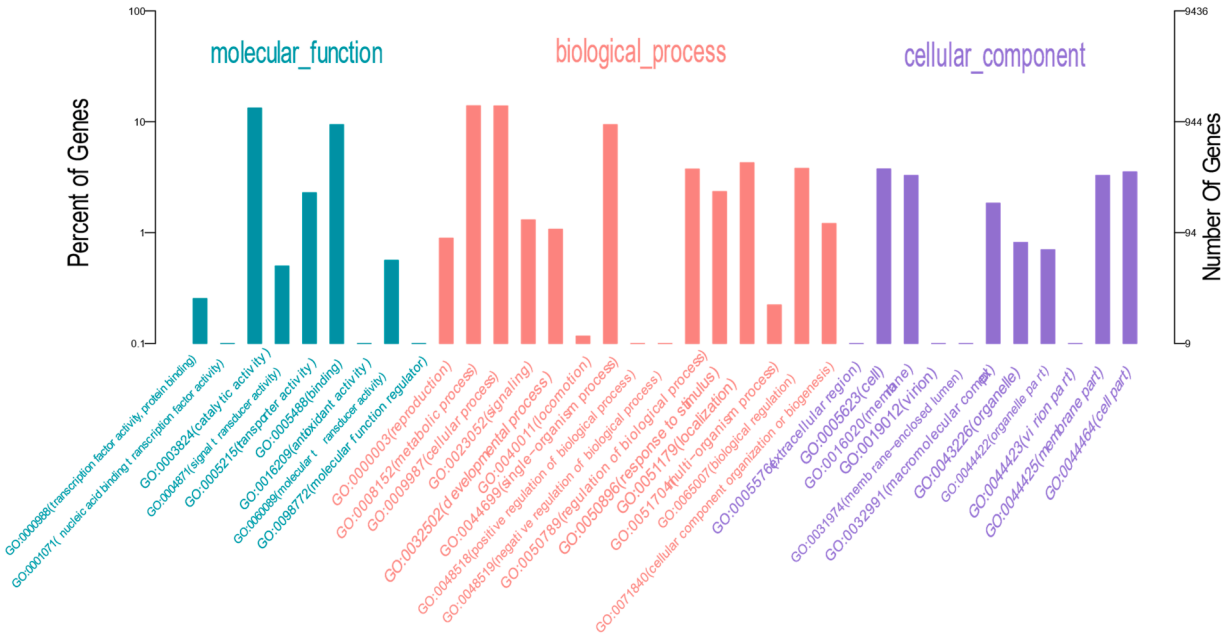

Fig. 4. Classification statistics of GO functions of genome-encoded proteins of *Bacillus cereus* BC4.

of some specific types of gene clusters was able to accurately identify gene clusters encoding all known pan-chemical classes of secondary metabolites.

In both bacterial and fungal genomes, secondary metabolite biosynthesis gene clusters may be quickly identified, annotated, and analyzed using AntiSMASH (Blin et al., 2019). *Bacillus cereus* could produce lots of secondary metabolites. Despite not being necessary for microbial growth, these complex chemical products are a significant source of medicines.

Fig. 7 shows the secondary metabolite gene cluster structure diagram of *Bacillus cereus* BC4 (Note:dark red represents main synthetic genes; pink represents secondary synthetic genes, blue represents transcription genes; green represents regulatory genes; gray represents other genes Gene.). Seven biosynthetic clusters were detected using anti-SMASH6.1.1, includingNRPS, Lanthipeptide-class-ii, Terpene and Beta-lactone (Table 3).

3.10. Analysis of genes related to chromium metabolism

The entire genome sequencing results of the *Bacillus cereus* strain BC4 were compared with the KEGG, COG, and GO databases by BLAST, and homology analysis was carried out to identify proteins associated to chromium metabolism. Sequence analysis showed that 14 chromium metabolism-related proteins were present in the *Bacillus cereus* BC4 genome. The protein associated with chromium resistance was probably 1 ChrA. There were 8 nitroreductases(NitR), 3 FMN-dependent NADH-azoreductases (AcpD), and 2 bifunctional riboflavin kinase/FAD synthetases (Ribf) (Table 2). The basic mechanism behind bacterial chromium metabolism involves the resistance to and reduction of Cr(VI), and the majority of the proteins involved in this mechanism belong to the CHR family (Díaz-Pérez et al., 2007), of which the ChrA protein has been investigated mostly and has the most chromium resistance (Ackerley et al., 2004).

Seventy oxidoreductase genes were also present in the sequence, as shown in Table S2, including four DAD(P)H-dependent oxidoreductase genes, three thioredoxin genes, three disulfide reductase genes, two Acyl-CoA dehydrogenase genes, one nitrite reductase gene, two LLM class flavin-dependent oxidoreductase genes, and one NADP-dependent isocitrate dehydrogenase gene (Table S2). These enzymes catalyze the production of large amounts of protons and electrons from the substrate, which further generate an electrochemical proton gradient across the membrane for transmembrane transport (He et al., 2011). Analysis of heavy metal resistance and its related genes

Heavy metal tolerance of *Bacillus cereus* BC4 and putative genes responsible for heavy metal resistance. The analysis revealed that the whole genome of *Bacillus cereus* BC4 contained several heavy metal resistance genes (Table S3), many arsenic metabolism-related proteins such as two arsenate efflux transporter proteins ACR3, one arsenic transporter protein ArsB, 13 arsenic resistance manipulator transcriptional regulators ArsR, and one arsenate reductase. Many copper resistance-related proteins such as copper efflux protein CopC, copper resistance protein CopD, copper dynamic homeostasis protein CutC and a multi-copper oxidase CopZ were present on *Bacillus cereus* BC4 whole genes. In addition, genes associated with Mn, Cd and Co resistance were found on *Bacillus cereus* BC4 whole genes (Table S3).

3.11. qRT-PCR analysis of related gene expression under different Cr(VI) concentrations

The findings from relative fluorescence quantitative PCR, as depicted in Fig. 8, reveal the inducible expression of the *chrA* and *nitR1* genes by Cr(VI) stress. Specifically, at a Cr(VI) concentration of 100 mg/L, the *chrA* gene's expression was elevated to 1.45 times that of the control, and the *nitR1* gene's expression surged impressively to 25 times the control level (He et al., 2011). Conversely, at a Cr(VI) concentration of 300 mg/L, the expression level of the *chrA* gene decreased to 4.93 times lower than the control, while the *nitR1* gene's expression was still 2.25 times higher than that of the control. The *chrA* gene, previously linked to Cr(VI) efflux in various studies, displayed augmented expression under a 100 mg/L Cr(VI) stress, suggesting the gene-encoded protein's critical role in Cr(VI) efflux. However, under a 300 mg/L Cr(VI) stress, its expression was suppressed compared to the control, hinting at possible inhibition by high Cr(VI) concentrations, potentially due to constitutive expression mechanisms (Zhu et al., 2019). The *nitR1* gene, associated with Cr(VI) reduction in several investigations, showed increased expression under 100 mg/L Cr(VI) stress, indicating the significant role of its encoded protein in Cr(VI) reduction. Even under the severe stress of 300 mg/L Cr(VI), its expression remained slightly elevated compared to the control. This suggests that although high concentrations of Cr(VI) may exert a suppressive effect on bacterial response mechanisms, the

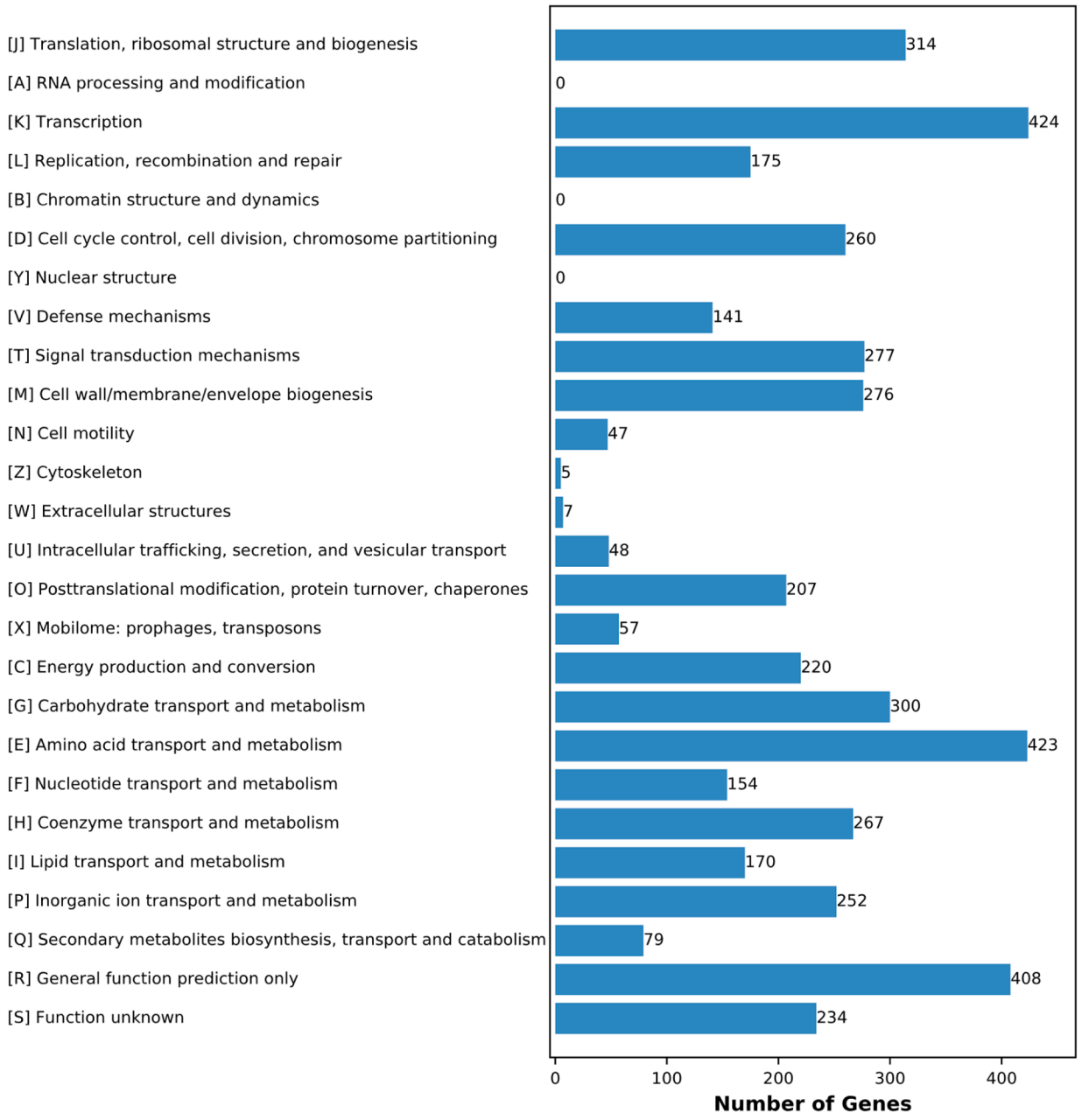

Fig. 5. Functional classification statistics of genome-encoded protein COG of *Bacillus cereus* BC4.

nitR1 gene still responds to some extent, which could imply its resilience or a key role in bacterial survival strategies under Cr(VI) stress.

4. Discussion

Harmful effects of Cr can be decreased by reducing Cr(VI) to Cr(III) (Liu et al., 2020; Pang et al., 2024). Research has demonstrated that several bacterial species have the ability to reduce Cr(VI). (Das et al., 2021; Bachate et al., 2012) The present study explored that *Bacillus cereus* BC4 was effective in eliminating Cr(VI), but the genes [Cr(VI) tolerance-related protein ChrA, flavonoid reductase Ribf, NAD(P)h-dependent azoreductase (AcpD) and nitroreductase (NfsA)] involved in the metabolism of Cr(VI) by this strain were still unknown.

The *chrA* gene, which is a member of the CHR superfamily, produces a transporter related to Cr(VI) resistance. The expression of genes related to hexavalent chromium [Cr(VI)] metabolism in *Bacillus cereus* BC4 is affected by several factors. Under Cr(VI) stress, the expression levels of the *chrA* and *nitR1* genes, in particular, were altered, probably through interactions with transcription factors or other regulatory proteins in the promoter region. *Cupravidus metallidurans* and *Pseudomonas aeruginosa*, were the first two proteins, which were used to study ChrA resistance mechanisms mediated by ChrA efflux proteins (Ackerley et al., 2004; Juhnke et al., 2002). The CHR superfamily proteins were essential for Cr resistance in chromium remediating bacteria. Numerous chromate resistance genes, including *chrA, chrB, chrC*, and *chrF*, were present in *O. tritici* and were found in the chromosomal DNA of chromium bacteria. For bacteria to develop high Cr(VI) resistance, the gene was necessary. The chromosome of the bacterium *Ralstonia metallidurans* has been shown to have the Cr(VI) resistance gene *chr2* (e.g. *chrB2, chrA2* and *chrF2* genes) (Juhnke et al., 2002). Bacterial plasmids, in addition to chromosomes, contained coding systems that shield cells from oxidative reaction brought on by Cr(VI) as well as genes for resistance to a variety of hazardous metals and metalloids (Gadd, 2010). Another illustration is the discovery that the *Ralstonia metallidurans*

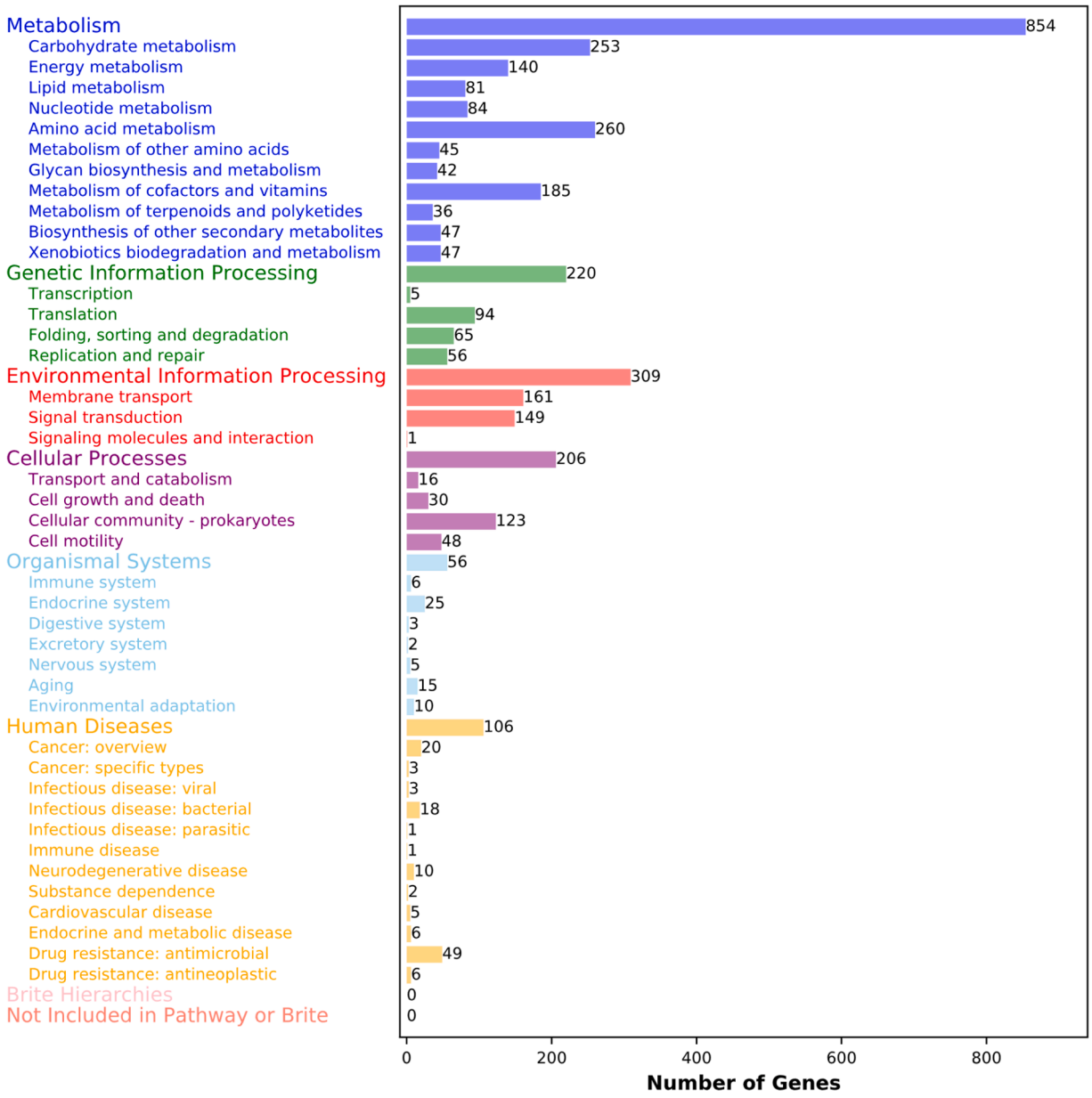

Fig. 6. Functional classification statistics of genome-encoded protein KEGG of *Bacillus cereus* BC4.

plasmid pMOL28 contains the genes for the chromate efflux pump ChrA as well as the chromate resistance-related proteins ChrC and ChrE (Juhnke et al., 2002). One of the *chrA* genes (WP_000429769) was 100 % homologous to the *chrA* gene in *Bacillus cereus* BC4 in this investigation, leading to the notion that the two organisms might share the same anti-chromium mechanisms.

In addition to the CHR superfamily, various oxidoreductases have been discovered in bacteria that catalyze the conversion of Cr(VI), including Cr(VI) reductase, nitroreductase (Kwak et al., 2003), Fe reduction enzymes, quinone reductases (Gonzalez et al., 2005), hydrogenases (Chardin et al., 2003), flavin reductases (Ackerley et al., 2004). Nitrate reductase (Nfs) shows Cr(VI) reduction activity under both aerobic and anaerobic conditions, Hexavalent chromium reductase directly converts toxic hexavalent chromium (Cr(VI)) to the less toxic trivalent chromium (Cr(III)). Iron reductases may be indirectly involved in the reduction of chromium, especially in certain microorganisms, and they may have broad substrate specificity. Quinone reductase may indirectly influence the environment for chromium reduction by catalyzing the reduction reaction of quinones. Hydrogenases are involved in hydrogen biological processes and may be relevant to chromium reduction under anaerobic conditions. Flavin reductase, on the other hand, reduces free radical loading by catalyzing the two-electron reduction of quinones and may provide electron donors or create suitable redox conditions for chromium reduction

Accordingly, it can be concluded that the mechanism of transport and reduction of hexavalent chromium by the strain BC4 is shown in Fig. 9, and the reduction of hexavalent chromium (Cr(VI)) by *Bacillus cereus* BC4 is achieved through a series of complex biochemical processes. This process involves the synergistic action of several key enzymes and genes, including ChrA transport proteins, nitroreductase (NitR), flavin monooxygenase (AcpD), and riboflavin kinase/FAD synthase (Ribf). These enzymes and proteins play a central role in the reduction of Cr(VI), and together promote the conversion of Cr(VI) to trivalent chromium (Cr(III)) through direct electron transfer, intracellular reduction pathways, and transmembrane transport mechanisms. In addition, genes related to multiple heavy metal resistance were identified in the genome of this strain, suggesting that *Bacillus cereus* BC4 is not only resistant to chromium, but may also have resistance and reducing

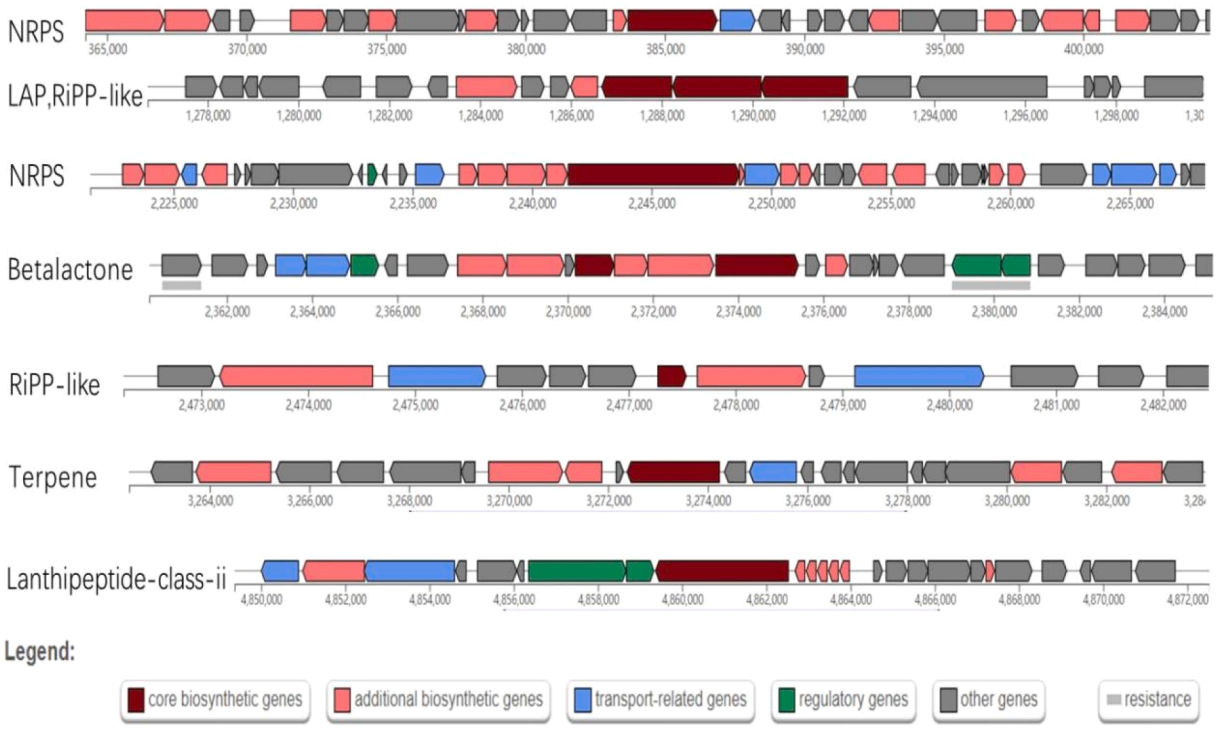

Fig. 7. Structure of secondary metabolite gene clusters of *Bacillus cereus* BC4.

Table 3
Secondary metabolite clusters identified in *Bacillus cereus* BC4 with antiSMASH6.1.1.

Region	Type	From	To	Most similar known cluster
1	NRPS	364,224	404,991	Thailanstatin A biosynthetic gene cluster (10 % of genes show similarity)
2	LAP,RiPP-like	1276,679	1300,186	_
3	NRPS	2221,516	2268,673	Bacillibactin biosynthetic gene cluster (46 % of genes show similarity)
4	Betalactone	2360,178	2385,416	Fengycin biosynthetic gene cluster (40 % of genes show similarity)
5	RiPP-like	2472,274	2482,540	_
6	Terpene	3262,380	3284,233	Molybdenum cofactor biosynthetic gene cluster (17 % of genes show similarity)
7	Lanthipeptide-class-ii	4849,367	4872,525	Cerecidin/CerecidinA1/CerecidinA2/CerecidinA3/CerecidinA4/CerecidinA5/CerecidinA6/CerecidinA7 biosynthetic gene cluster (94 % of genes show similarity)

ability to other heavy metals such as arsenic, copper, manganese and cadmium. The up-regulation of *chrA* and *nitR1* gene expression under low Cr(VI) stress was further confirmed by qRT-PCR analysis, implying that these genes play important roles in Cr(VI) resistance and reduction.

In conclusion, the *Bacillus cereus* BC4, isolated from contaminated soil, demonstrated a remarkable ability to reduce Cr(VI) levels. Specifically, under culture conditions of pH 8, a temperature of 37 °C, and a shaking speed of 120 rpm, it successfully reduced to 98.6 % of Cr(VI) in LB medium initially containing 300 mg/L. Through further exploration of its genetic material, this bacteria's genome was sequenced to uncover mechanisms behind its resistance and reduction capabilities toward Cr

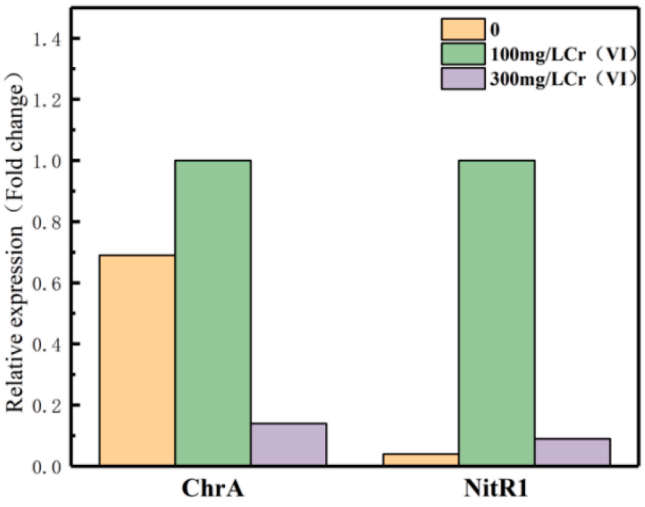

Fig. 8. Relative fluorescence quantitative PCR results of *Bacillus cereus* BC4.

(VI). A key discovery was the presence of the Cr(VI) tolerance-associated protein ChrA within *Bacillus cereus* BC4. Additionally, proteins related to Cr(VI) reduction, including flavoprotein reductase (Ribf), NAD(P)H-dependent nitroreductase (NfsA), nitroreductases (NitR), and azoreductase (AcpD), were identified. The results from relative fluorescence quantitative PCR highlighted a significant upregulation in the expression of the nitroreductase gene *nitR1*, and a moderate increase in *chrA* gene expression under conditions of low Cr(VI) stress. This suggests that the proteins encoded by these genes are instrumental in the strain's ability to remove Cr(VI). Moreover, the genomic sequencing of *Bacillus cereus* BC4 revealed genes conferring resistance to other heavy metals, including arsenic (As), copper (Cu), cadmium (Cd), manganese (Mn), and cobalt (Co). The presence of these genes not only underscores the strain's potential in bioremediation efforts for environments polluted with various heavy metals but also contributes to the foundational

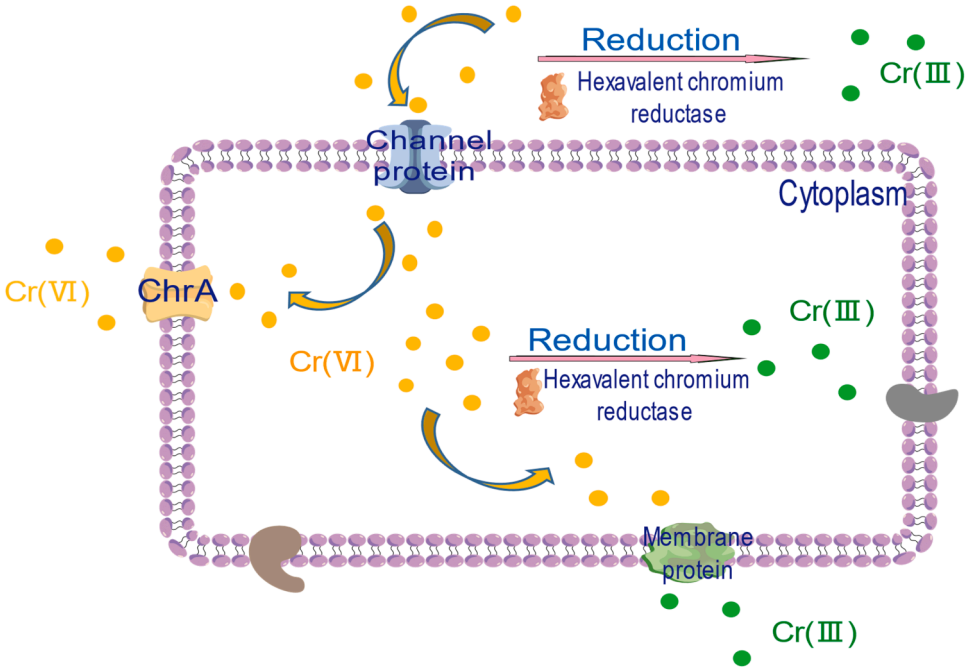

Fig. 9. Mechanism of Cr(VI) remediation accomplished of *Bacillus cereus*BC4.

knowledge required for microbial informatics approaches to treating hexavalent chromium pollution. This study therefore provides valuable insights into the bioremediation capabilities of *Bacillus cereus* BC4, highlighting its potential as a highly effective tool in the cleanup of environments contaminated with a variety of hazardous substances.

5. Conclusion

Bacillus cereus BC4 was effective in reducing hexavalent chromium concentration under specific conditions, and its genome sequencing revealed resistance genes and biopolymer synthesizing ability to a variety of heavy metals. qRT-PCR showed that the expression of *chrA* and *nitR1* genes was up-regulated under chromium stress, suggesting their key roles in chromium resistance. This study not only deepens our understanding of the potential of the strain BC4 in chromium pollution remediation and environmental restoration, but also submits the complete genomic data to GenBank, which provides valuable resources for future research and applications.

Consent for publication

Not Applicable.

CRediT authorship contribution statement

Yajun Cai and Qi-an Peng conceived the idea. Qi-an Peng obtained funds. Zhiyi Liu, Yubing Cai, Xu Chen, Yan Cang, and Jialiang Yu performed experiments. Yajun Cai, Qi-an Peng and Muhammad Shaaban analyzed data. Yajun Cai, Qi-an Peng and Muhammad Shaaban wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2025.100388.

Data availability

Data will be made available on request.

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