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Comparative genomic analysis of Citrobacter sp. XT1-2-2 reveals insights into the molecular mechanism of microbial immobilization of heavy metals

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Abstract

Background: In our previous study, *Citrobacter* sp. XT1-2-2 was isolated from high cadmium-contaminated soils, and demonstrated an excellent ability to decrease the bioavailability of cadmium in the soil and inhibit cadmium uptake in rice. In addition, the strain XT1-2-2 could significantly promote rice growth and increase rice biomass. Therefore, the strain XT1-2-2 shows great potential for remediation of cadmium -contaminated soils. However, the genome sequence of this organism has not been reported so far.

Results: Here the basic characteristics and genetic diversity of the strain XT1-2-2 were described, together with the draft genome and comparative genomic results. The strain XT1-2-2 is 5040459 bp long with an average G + C content of 52.09%, and contains 3787 protein-coding genes and 129 RNA genes. All genes of a complete set of sulfate reduction pathway and various putative heavy metal resistance genes in the genome were identified and analyzed.

Conclusions: These analytical results provide insights into the molecular mechanism of microbial immobilization of heavy metals.

1. Background

The *Citrobacter* genus belongs to the family Enterobacteriaceae of the class Gammaproteobacteria [1], and was introduced in 1932 by Werkman and Gillen [2], which is a distinct group of Gram-negative, non-spore-forming, rod-shaped, facultative anaerobic and mobile bacteria [1, 3]. The *Citrobacter* genus typically utilizes citric acid as the primary carbon source [4]. *Citrobacter* species is commonly found in soil, water, sewage and food, sometimes exists as a normal flora in the gastrointestinal tract, also in human and animal feces, and sometimes as opportunistic pathogens isolated from clinical samples [5, 6].

Citrobacter sp. XT1-2-2 was isolated from high Cd-contaminated paddy soil. In our previous study, we found that the strain XT1-2-2 could tolerate a variety of heavy metals, and showed remarkable removal efficiency of Cd^{2+} in the solution compared with controls. Meanwhile, the strain could decrease the bioavailability of Cd in the soil and inhibit Cd uptake in rice plants. In addition, the strain could significantly promote rice growth and increase rice biomass [7]. These effects are mainly due to the strain's ability to reduce sulfate ($SO4^{2-}$) to sulfide ions (S^{2-}), and then sulfide ions (S^{2-}) can combine with cadmium ions (Cd^{2+}) existing in the soil to produce cadmium sulfide (CdS) precipitation, thereby converting the highly active cadmium ions (Cd^{2+}) into residual cadmium sulfide (CdS), and then reduces the absorption and transport of cadmium by rice [8, 9]. Therefore, these characteristics made the strain XT1-2-2 strong potential for application to remediate Cd-contaminated paddy soils. However, the genome sequence and basic properties of this organism have not been reported so far. Here we report the high quality draft genomic information of the strain XT1-2-2 and conduct comparative genomic analysis with the other relevant reference sequenced genomes.

2. Results

2.1 Organism classification and characteristics

The strain XT1-2-2 is Gram-negative, facultatively anaerobic, motile and rod-shaped (Fig. 1). The colonies are ciucular, smooth and opaque with a regular slick edge on SRB agar plates [8]. The basic characteristics and classification of the strain XT1-2-2 are shown in Table S1. The results of previous studies showed that the strain XT1-2-2 exhibited high resistance to a variety of heavy metals, and the MIC of the strain XT1-2-2 for Cd^{2+} was as high as 400 mg/L. The phylogenetic tree of *Citrobacter* sp. XT1-2-2 was constructed based on the 16S rRNA gene sequences via neighbor-joining method, and according to the results of phylogenetic analysis, the strain XT1-2-2 was most closely related to *Citrobacter werkmanii* strain BF-6 and *Citrobacter youngae* ATCC 29220 (Fig. S1).

2.2 Genome sequencing, annotation and features

The strain XT1-2-2 was selected for sequencing particularly due to its multiple heavy metals resistance and heavy metal removal ability. Genome sequencing was performed by Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). The project information is summarized in Table S2. The constructed standard shotgun library generated 165575 reads totaling 1164774594 bp and an average length of 7034.7 bp. The total size of the genome is 5,040,459 bp with 52.09% G + C content (Fig. 2). The genome properties and statistics are shown in Table 1. From a total of 4816 genes, 3787 (78.6%) are protein coding genes, and 110 predicted RNA genes, including 83 tRNA and 25 rRNAs. In addition, 4383 (91.0%) genes are distributed into COG functional categories (Fig. 3).

Table 1
The genome properties and statistics of *Citrobacter* Sp. XT1-2-2

Attribute	Value
Genome size (bp)	5040459
CDS No.	4815
G + C Content(%)	52.09
tRNA No.	83
Type of tRNAs No.	21
rRNA No.	25
Gene No.	4816
Gene total length(bp)	4459947
Gene average length(bp)	926.07
Gene density(kb)	0.96
GC content in gene region(%)	53.11
Gene/Genome(%)	88.48
Intergenetic region length(bp)	580512
GC content in intergenetic region(%)	44.19
Intergenetic length/Genome(%)	11.52
Total reads num	165575
Average length of reads(bp)	7034.7
Bases in all scaffolds(bp)	5040459
Scaffold N50(bp)	5040459
Scaffold N90(bp)	5040459
CRISPR-Cas No.	2
Total lengthof tandem repeat(bp)	6530
Tandem repeat /Genome(%)	0.13
Protein-coding genes	3787
Genes No. of Cellular Component	2053
Genes No. of Molecular Function	3098

Attribute	Value
Genes No. of Biological Process	3098
Genes assigned to COGs	4383
Genes with Pfam domains	4294
Genes with transmembrane helices	1170
Genes with transport proteins	1195
Genes with signal peptides	416

2.3 Identification of sulfate reduction pathway

According to the KEGG prediction analysis, the strain XT1-2-2 contains all genes of the complete set of sulfate reduction pathway (Fig. 4), including cysA, cysC, cysD, cysH, cysI, cysJ, cysN, cysP, cysU, cysW. which provides the genomic basis for the strain to reduce sulfate ($SO4^{2-}$) to sulfide (S^{2-}) to form CdS precipitation, thereby reducing the uptake and transport of Cd^{2+} by rice.

2.4 Identification of heavy metal resistance genes

The results of previous studies showed that the strain XT1-2-2 could tolerate a variety of heavy metals (Cd^{2+}, Pb^{2+}, Zn^{2+}, Mn^{2+} and Cr^{6+}) and the removal rate of Cd^{2+} in solution is as high as 82.3 ± 2.1% within 240 min [7]. These results suggest that the strain XT1-2-2 has developed many evolutionary strategies to adapt the complex heavy metal pollution environment. According to the results of genome annotation, the strain XT1-2-2 contains multiple putative functional proteins, which are related to heavy metal resistance, including transporters, resistance proteins and metal reductases, and so on (Fig. 5).

2.5 Features of the core and pan-genomes

In order to assess genetic diversity, we constructed *Citrobacter* genus core and pan genomes and compared the gene content of *Citrobacter* sp. XT 1-2-2 with other relevant reference strains (Fig. 6). From the alignment results, 13,614 gene families were found in 16 genomes, of which 2,449 genes constitute the core genome. The functional categories of the core gene families were further determined via the Cluster of Orthologous Group (COG) assignments among all the related species. The results showed that the core gene family presented an uneven distribution among functional categories (Fig. 3).

2.6 Comparative genomics analysis

The amino acid sequences of the involved twenty species were aligned via the OrthoMC, and a certain threshold (E-Value: 1e-5, Percent Identity Cutoff: 0, Markov Inflation Index: 1.5) was selected for similarity clustering to obtain homologous genes. With the help of Venn diagram, the common and unique

homologous genes between species are displayed intuitively. The strain XT1-2-2 shares 2285 proteins with the other genomes and has 342 specific proteins. The 2285 core genes include the genes in the whole sulfate reduction pathway and most of the heavy metal resistance genes (Fig. 7).

3. Discussion

In this study, the complete genome of *Citrobacter* sp. XT1-2-2 was sequenced and comparative genomics analysis was also conducted with the other relevant reference sequenced genomes. In our previous study, the strain XT1-2-2 was isolated from high Cd-contaminated soils, and demonstrated an excellent ability to decrease the bioavailability of Cd in the soil and inhibit Cd uptake in rice. In addition, the strain XT1-2-2 could significantly promote rice growth and increase rice biomass. However, the genome sequence of this organism has not been reported so far.

The antigenic system of the Bethesna-Ballerup group bacteria was established by West and Edwards in 1954 [10]. This group of bacteria is now called *Citrobacter freundii* [11]. So far, *Citrobacter* genus contains eleven species: *Citrobacter freundii*, *Citrobacter koseri*, *Citrobacter amalonaticus*, *Citrobacter farmeri*, *Citrobacter youngae*, *Citrobacter braakii*, *Citrobacter werkmanii*, *Citrobacter sedlakii*, *Citrobacter rodentium*, *Citrobacter* genomospecies 10, *Citrobacter* genomospecies 11 [12, 13]. According to the results of phylogenetic analysis, the strain XT1-2-2 was most closely related to *Citrobacter werkmanii* strain BF-6 and *Citrobacter youngae* ATCC 29220 (Fig. S1). According to the physicochemical properties of these strains, some *Citrobacter* species immobilized biofilms were used to bioremediate heavy metal contaminated soils through an acid-type phosphatase enzymatic activity or their ability to accumulate heavy metals [14-16]. In this study, genome analysis of the strain XT1-2-2 revealed all genes of a complete set of sulfate reduction pathway according to the KEGG analysis (Fig.4). The occurrence of metabolic pathways involves the following steps: (1) Sulfate ($SO4^{2-}$) from outside is taken up into cells by putative sulfate transporter CysPUWA; (2) Sulfate ($SO4^{2-}$) entering the cell is first acetylated to adenylylsulphate (APS) by sulfate adenylyltransferases CysN and CysD; (3) The resulting APS is then phosphorylated to phosphoadenylyl-sulphate (PAPS) by the APS kinase CysC; (4) The resulting PAPS is further reduced to sulfite ($SO3^{2-}$) by PAPS reductase CysH; (5) The resulting sulfite ($SO3^{2-}$) is finally reduced to sulfide (S^{2-}) by sulfite reductase CysIJ [9]. The reason why the strain XT1-2-2 has a significant effect of removing cadmium is mainly because the strain generates sulfide (S^{2-}) via the sulfur metabolism pathway, which can combine with Cd^{2+} in the soil to form the precipitated CdS, thereby reducing the uptake and transport of cadmium in the soil by rice plant.

Meanwhile, the strain XT1-2-2 also revealed various genes responsible for multiple heavy metal resistance (Fig. 5), which provided the genomic basis for the strain to adapt to the external complex harmful environment. CzcD is involved in resistance to the heavy metals Cd^{2+}, Zn^{2+} and Co^{2+} [17]. The membrane transporter ChrA is responsible for the efflux of intracellular Cr(VI) from the cell [18]. Heavy metal-transporting ATPase (ZntA) is responsible for the efflux of Pb^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} [19]. The metal ABC transport system (ZnuABC) are involved in Zn^{2+} uptake [20]. ArsB, ArsC, and ArsH proteins are

involved in the functions of arsenical pump membrane protein, arsenate reductase and arsenical resistance protein, respectively [21]. Cus copper resistance system consists of CusCBA efflux pump, CusF periplasmic protein and CusS regulatory protein [22]. Mercury transport system (*mer* operon) encodes a group of proteins consisting of MerR mercury regulatory proteins, MerT, MerC, MerP mercury transport proteins and MerA, MerD, MerE mercury resistance proteins [23]. The Co^{2+} ECF transporter complex is involved in Co^{2+} resistance and transmembrane transport [24].

The analysis of the core and pan genomes showed an uneven distribution among functional categories (Fig. 3). There were several notable differences in the numbers of genes, such as amino acid transport and metabolism (category E), transport and metabolism of carbohydrates (category G), translation (category K) and inorganic ion transport and metabolism (category P). In particular, this difference in the number of genes belonging to the same COG category was mainly reflected in transport and metabolism [1]. For KEGG annotations, two gene functional categories were enriched in core gene families including metabolism and environmental information processing (Fig. 8). It may be due to the fact that the signal transduction system faces the complex and changeable external environment, and the metabolic pathway needs to respond quickly to adapt to the environmental changes.

4. Conclusions

Results of comparative genomic analysis from *Citrobacter* sp. XT1-2-2 revealed correlations between genotype and phenotype. Genome analysis revealed all genes of a complete set of sulfate reduction pathway according to the KEGG analysis, which provides the genomic basis for the strain to reduce sulfate ($SO4^{2-}$) to sulfide (S^{2-}) to form CdS precipitation, thereby reducing the uptake and transport of Cd^{2+} by rice plants. Meanwhile, the strain also revealed various genes responsible for multiple heavy metal resistance, which provided the genomic basis for the strain to adapt to the external complex harmful environment. These analytical results provide insights into the molecular mechanism of microbial immobilization of heavy metals.

5. Materials And Methods

5.1 Bacterial strain and DNA extraction

The strain XT1-2-2 was initially isolated from high Cd- contaminated paddy soils (~ 220 mg/kg) in Liuyang city, Hunan Province, China (28°01’N, 113°34’E). Based on previous morphological and molecular characterization, the strain XT1-2-2 was identified as the genus *Citrobacter* sp.. The genomic DNA of the strain XT1-2-2 was extracted by QIAamp DNA Mini Kit (Qiagen, CA, USA) according to the manufacturer’s protocol.

5.2 Genome sequencing and assembly

The whole genome sequence of the strain XT1-2-2 was obtained via the Illumina Hiseq×10 and Pacbio platforms, with a depth of ~ 100-fold coverage in both platforms. The previously extracted genomic DNA

was randomly fragmented through Covaris or Bioruptor method. Fragmented DNA was purified by the QIAquick Nucleotide Removal Kit (Qiagen, Crawley, United Kingdom). Sequencing adaptors were ligated to A-tailed 3'ends according to the manufacturer's instructions. A library for Illumina Paired-End sequencing was prepared. The prepared libraries were sequenced by Illumina Hiseq×10. In order to construct the high quality complete genome of the strain XT1-2-2, the prepared genome was also sequenced by the Pacbio platform. The resulting reads were *de novo* assembled with the help of SOAPdenovo v1.05. The genome was annotated using the NCBI Pro-karyotic Genome Annotation Pipeline (PGAP), and genes were identified by the gene caller GeneMarkS.

5.3 Identification of gene orthologous groups

OrthoMC was exploited to determine orthologous families in the pan-genome with default parameter (E-Value:1e-5, Percent Identity Cutoff:0, Markov Inflation Index:1.5). The single-copy core gene and pan gene were extracted with the help of the OrthoMC (http://www.orthomcl.org/common/downloads/software/v2.0/). Their nucleotide sequences were extracted on the basis of protein ID.

5.4 Phylogenetic analysis

Based on the identified gene homology clusters, a total of 2285 single-copy homology core genes were mined, which were shared to each genome. The nucleotide sequences of the identified single-copy core genes were aligned and analyzed by MAFFT. These orthologous core genes were classified into 20 types. The phylogenetic relationship of the strain XT1-2-2 to the other related members was predicted via the maximum likelihood (ML) algorithm in PhyML. The RaxML was used to construct the phylogenetic tree.

5.5 Core and pan-genome analysis

Core and pan-genome analyses were manipulated by the 16 genome sequences of *Citrobacter* related species, respectively. The regression analysis of the core gene cluster curves was performed via a weighted least square regression.

5.6 Gene functional category

The functional category of the core gene families was analyzed and classified by different database (COG/GO/KEGG). The numbers of corresponding proteins were computed for each term of COG/GO/KEGG.

The main biological functions of different proteins were determined by functional enrichment analysis, and then the resulting results were visualized by GraphPad Prism 7.0.

Abbreviations

KEGG: Kyoto Encyclopedia of Genes and Genomes

GO: Gene Ontology

COG: Cluster of Orthologous Groups of Proteins

MAFFT: multiple alignment program for amino acid or nucleotide sequences

Declarations

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Availability of data and materials

The genome sequence of *Citrobacter* sp. XT1-2-2 has been deposited in GenBank under the BioSample number SAMN28157541.

Contributions

SS, YL,WC and MZ wrote manuscript. XW, YW and SW collected and analyzed data. DD and ZG interpreted the data and reviewed manuscript. ZL and ZF edited the manuscript. All authors read and approved the final manuscript.

Ethics approval 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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Figures

Figure 1

Scanning electron micrograph of *Citrobacter* sp. XT1-2-2.

Figure 2

A graphical circular map of *Citrobacter* sp. XT1-2-2.

From outside to center, rings 1, 4 show protein-coding genes colored by COG categories on forward/reverse strand; rings 2, 3 denote genes on forward/reverse strand; ring 5 shows G+C % content plot; ring 6 shows GC skew; the innermost ring shows the marker of genome size.

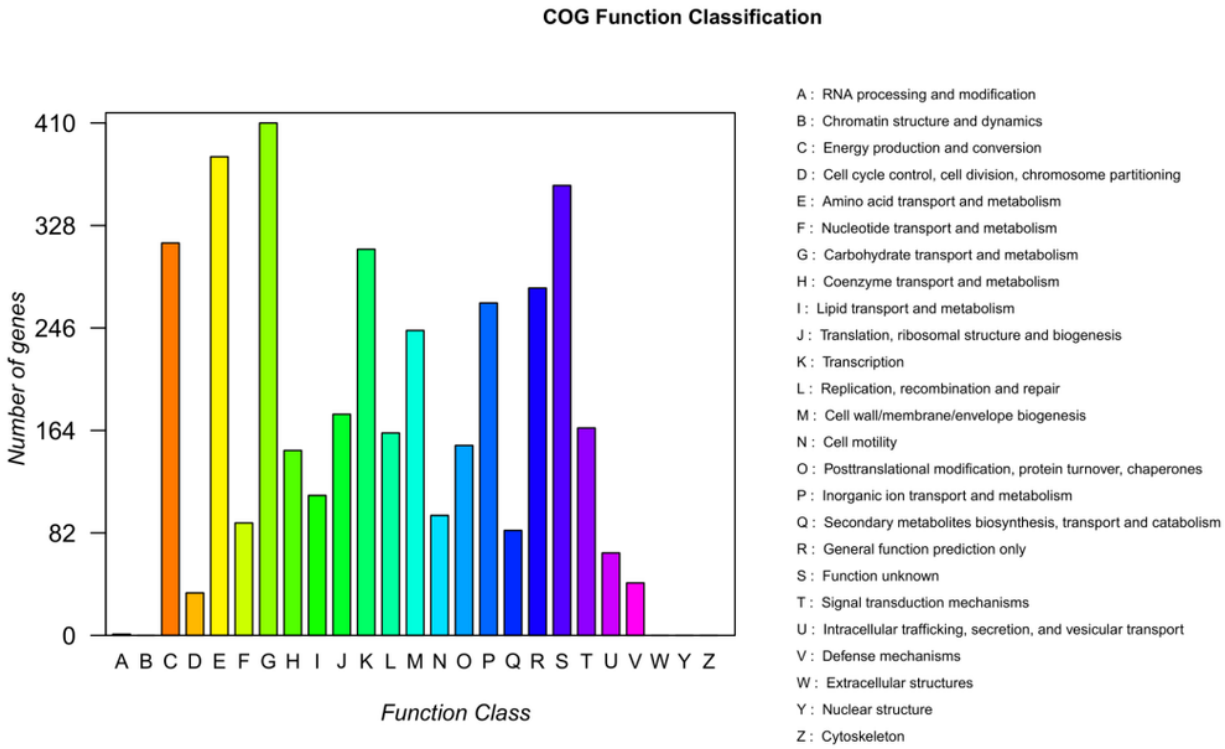

Figure 3

Number of genes associated with the 20 general COG functional categories.

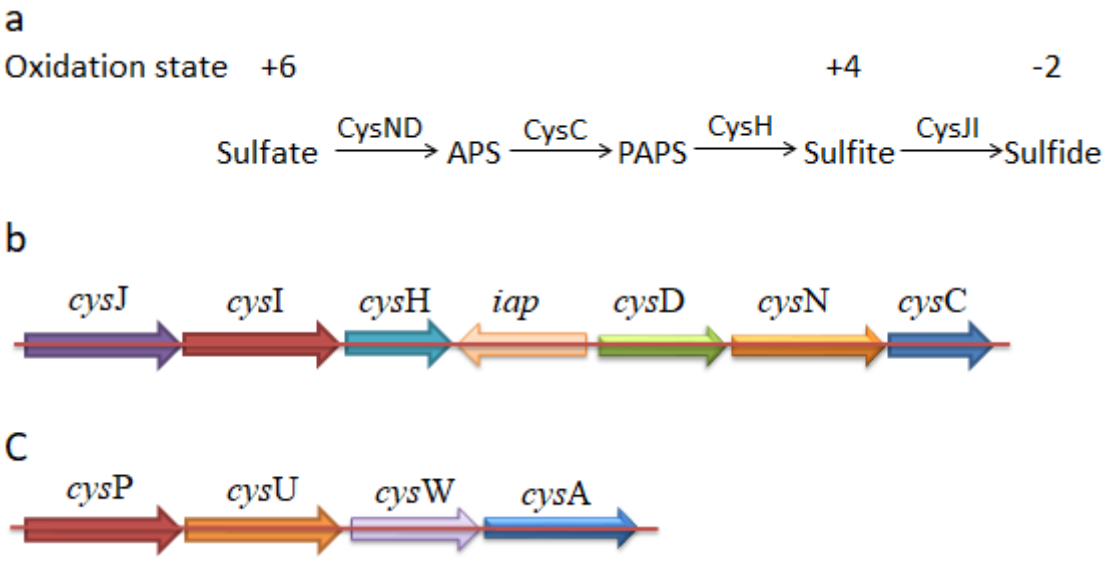

Figure 4

Putative sulfur metabolism pathway and assimilatory sulfate reduction gens in *Citrobacter* sp. XT1-2-2. a Putative sulfur metabolism pathway. b Assimilatory sulfate reduction gens in *Citrobacter* sp. XT1-2-2. c

Putative sulfate transporter CysPUWA.

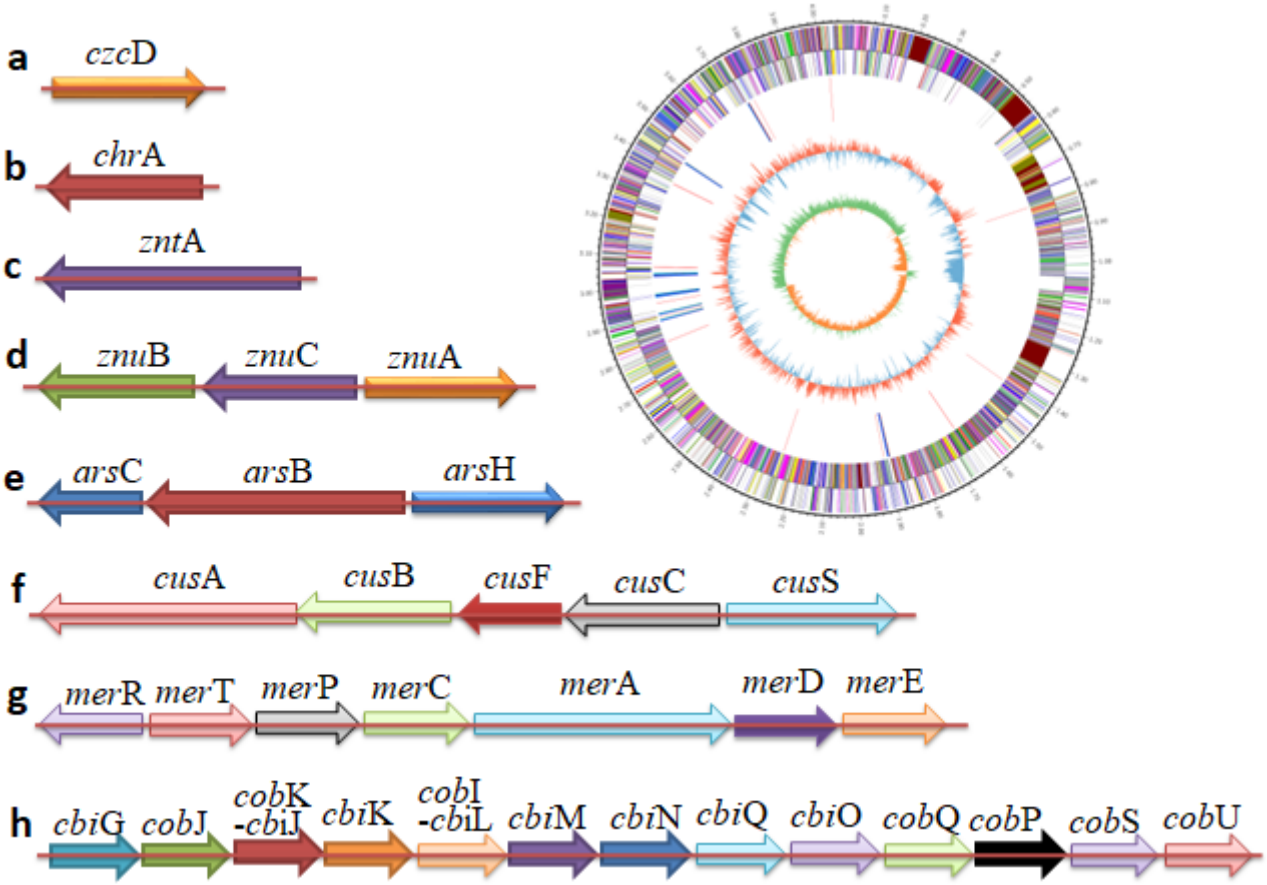

Figure 5

Heavy metal resistance genes distributed in *Citrobacter* sp. XT1-2-2. a Zinc or cadmium transporter, b Chromate transporter, c Zinc/ cadmium /mercury /lead-transporting ATPase, d Zinc ABC transporter permease, e Arsenical resistance protein, f copper resistance system, g Mercury transport system, h Cobalt ECF transporter complex.

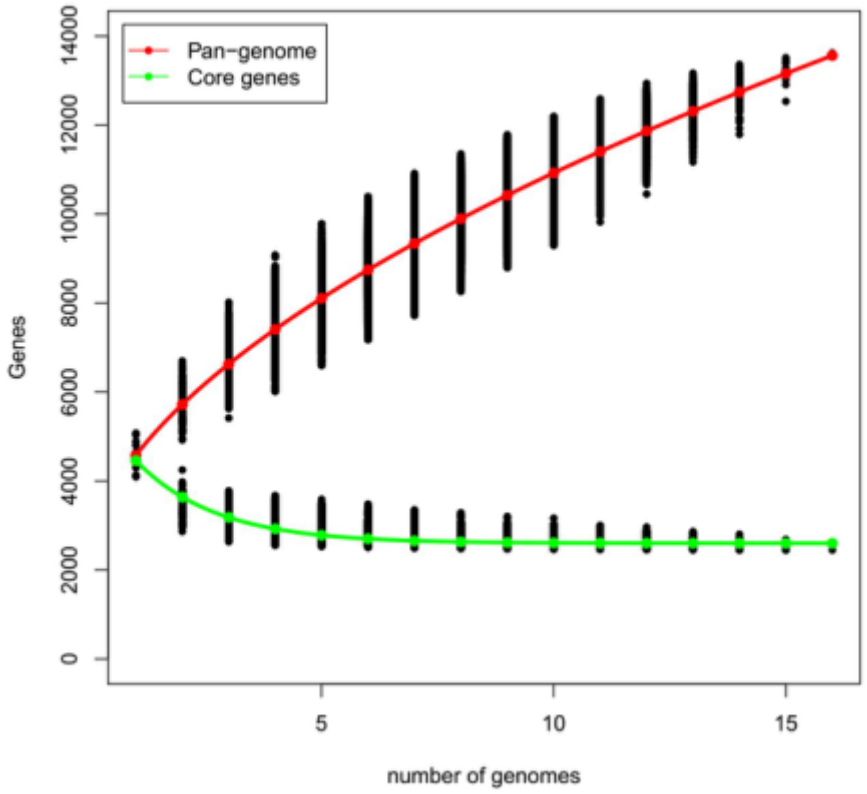

Figure 6

The *Citrobacter* core and pan-genome plotted were constructed for 16 genome sequences of *Citrobacter* related species.

Figure 7

The Venn diagram depicting the core and unique genes between *Citrobacter* sp. XT1-2-2 and other 19 relevant reference species.

Figure 8

Distribution of functional catalogs of core genes in *Citrobacter* sp. XT1-2-2 after KEGG annotation.

Supplementary Files

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- Supplementarymaterials.doc