DOI: 10.53660/CLM-3555-24J20

Mining a bioremediation genetic toolbox in the novel environmental bacteria *Enterobacter cloacae* strain amazonensis

Explorando uma ferramenta genética de biorremediação na nova bactéria ambiental *Enterobacter cloacae* cepa amazonensis

Received: 21-04-2024 | Accepted: 25-05-2024 | Published: 31-05-2024

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ABSTRACT

The rise of environmental pollution is a threat to planetary and human health. Microbes encode a wide range of enzymes with sophisticated catalytic properties to mitigate toxic chemicals from nature. Bioprospecting environmental bacteria can be a powerful resource for improved enzymes to optimize bioremediation processes. Here, we present a promising genetic toolbox sequenced from a novel multi-resistant bacterial strain isolated from industrial wastewater and sewage-contaminated stream in the city of Manaus, Amazonas, Brazil. We report *Enterobacter cloacae amazonensis* as a highly heavy-metal resistant and antibiotic multi-resistant strain. Functional and comparative genomics of the *E. cloacae* strain *amazonensis* draft genome revealed the annotation of 104 genes encoding proteins involved in the metabolism of copper, cobalt, zinc, cadmium, chromium, mercury and arsenic, as well as a plethora of broad-spectrum resistant genes. As a proof of concept, we characterized a plasmid mobile element from the *amazonensis* strain, pEN_*Amazonensis*, in the model biotechnological workhorse *E. coli*, leading to the robust acquisition of mercury and antibiotic resistance. Here, we highlighted the potential of bioprospecting genetic novelty from environmental bacteria through comparative genomics, paving the way for a genetic toolbox for bioremediation processes.

Keywords: mercury; environmental bacteria; bioprospecting.

RESUMO

O aumento da poluição ambiental é uma ameaça ao planeta e à saúde humana. Os microrganismos codificam uma ampla gama de enzimas com propriedades catalíticas sofisticadas para mitigar produtos químicos tóxicos da natureza. A bioprospecção de bactérias ambientais pode ser um recurso poderoso para o melhoramento de enzimas e otimização dos processos de biorremediação. Aqui, apresentamos uma ferramenta genética promissora sequenciada a partir de uma nova cepa bacteriana multirresistente isolada de águas residuais industriais e córregos contaminados com esgoto na cidade de Manaus, Amazonas, Brasil. Relatamos *Enterobacter cloacae amazonensis* como uma cepa altamente resistente a metais pesados e multirresistente a antibióticos. A genômica funcional e comparativa do genoma preliminar da cepa *E. cloacae amazonensis* revelou a anotação de 104 genes que codificam proteínas envolvidas no metabolismo de cobre, cobalto, zinco, cádmio, cromo, mercúrio e arsênio, bem como uma infinidade de genes resistentes de amplo espectro. Como prova de conceito, caracterizamos um plasmídeo, elemento móvel da cepa amazonensis, pEN_Amazonensis, um carreador no modelo biotecnológico para *E. coli*, levando à aquisição robusta de resistência ao mercúrio e a antibióticos. Aqui, destacamos o potencial da bioprospecção de novidades genéticas a partir de bactérias ambientais por meio da genômica comparativa, abrindo caminho para uma nova ferramenta genética para processos de biorremediação.

Palavras-chave: mercúrio; bactérias ambientais; bioprospecção.

INTRODUÇÃO

The degradation of natural ecosystems as an outcome of industrialization and urbanization led to widespread and high-level dissemination of heavy metals in the food chain. Among a range of heavy metals, mercury comes to light for its extremely toxic and bioaccumulative properties (ZHANG, *et al.*, 2014; MISHRA *et al.*, 2019; OKEREAFOR *et al.*, 2020). Through biochemical processes, Hg^{2+} is converted into methylmercury, presenting a strong affinity to the sulfhydryl group in proteins. This ability to bind to cellular components promotes the biomagnification of mercury, in which the concentration of Hg increases in higher trophic levels in the food chain, threatening the fauna, flora, and human health (AZEVEDO-SILVA *et al.*, 2016; ASHE, 2012).

Through environmental selective pressure, microbes evolved sophisticated adapting mechanisms to heavy metals, such as efflux pumps, chelating, precipitation, and enzymatic reduction. Resistance genes are often found in complex genetic clusters encoding proteins regulating gene expression, binding, transporting, and enzymes catalyzing detoxification reactions. Among a diversity of genetic machinery, the mer operon is one of the best characterized biological systems for mitigating heavy metal toxicity. Microbial mercury metabolism is catalyzed by a key enzyme, MerA, which reduces Hg (II) to volatile Hg (0). The mer operon is regulated by MerR, acting as a transcriptional repressor or activator in the absence and presence of Hg (II) which regulates the expression of merTPCADE in bidirectional expression machinery´. Synthetic biology and genetic engineering have been designing and building bioremediation and biosensing devices based on the mer operon functions, and it's been the foundation to innovative technologies able to clean mercury contamination (ZHANG *et al.*, 2021).

As novel technologies advance, driven by the engineering of genetic systems, bioprospecting, and mining for better enzymes paves the way to improved biotechnological processes. Next-generation sequencing has enabled big data sets in which mining has mentioned a vast number of microbes with biodegradation potential. Enzymes that were functionally sourced from public databases are the foundation of a wide variety of technologies. The integration of several computational techniques in silico are applicable to studies, genomics, metagenomics, metabolomics, transcriptomics, proteomics and biodegradation network pave the way for heavy metal bioremediation (VON NETZER *et al.*, 2018; PUROHIT *et al.*, 2018).

The Igarapé do 40 is one of the urban streams most impacted by environmental degradation in the Brazilian Amazon region. Unregulated industrial disposal from manufacturing in the Distrito Industrial and domestic sewage are the main drivers of the high level of pollutants contaminating the wastewater stream (PUROHIT *et al*., 2018; MORAES *et al*., 2018).

We believe such environmental pressure has enabled the evolution of powerful genetic resources. Sequencing and characterizing them can assist in developing new bioremediation technologies. Here, we conducted (1) microbial resistance assays with the *E. cloacae amazonensis*, a novel strain isolated from *Igarapé do 40*; (2) comparative genomics analysis of the genes encoding heavy metal detoxification; (3) a proof-of-concept experiment characterizing *E. cloacae amazonensis* high resistant plasmid, pEN_Amazonensis, in the *E. coli* DH5a lineage to demonstrate bioremediation activity. Here, our goal is to characterize the heavy metal resistance profile of the *E. cloacae amazonensis*, highlighting the potential of using comparative genomics in environmental bacteria to reveal novel genes for bioremediation.

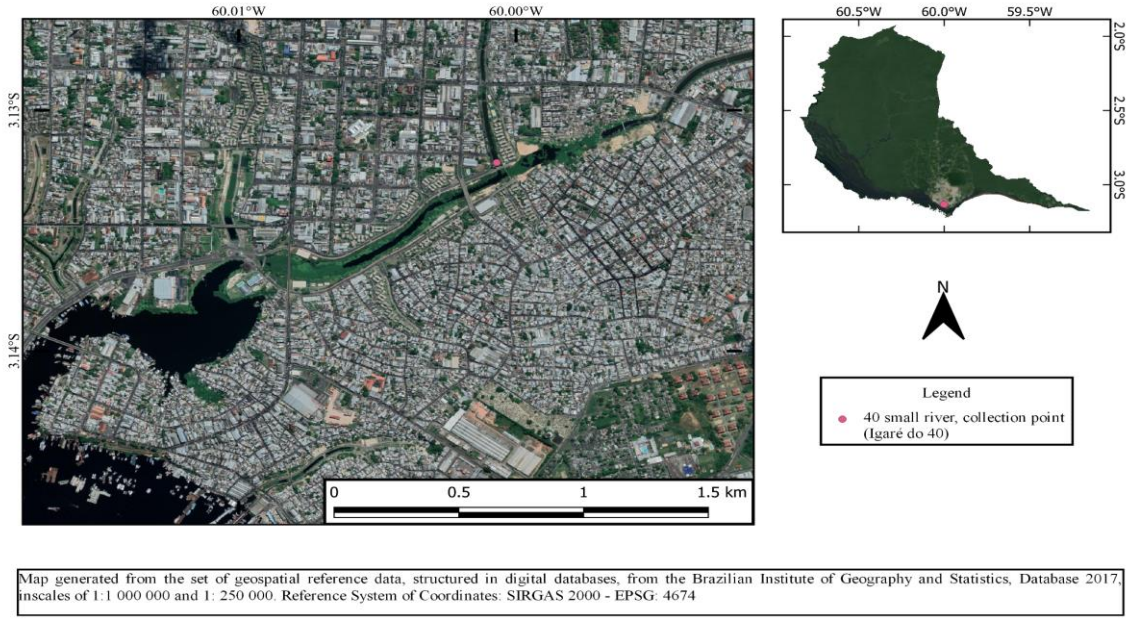
MATERIAL AND METHOD

Sample collection and microbiology assays

Water samples were collected from Igarapé do 40 (3º.07’55.8” S 60º.00’02.5” W), a contaminated wastewater stream located in Manaus, Amazonas, Brazil (Fig. 1). Here, we selected Igarapé do 40 due to its industrial wastewater and domestic sewage exposure. We believe it is a promising reservoir for heavy metal resistance and antimicrobial genes.

Samples were collected in 50mL sterile Falcon tubes. Serial dilution from 10-1 to 10-5 was performed and cultured in solid Luria Bertani plates at 30ºC. Isolated colonies were cultivated in selective media containing 20 to 100 µg/mL of mercury chloride (HgCl2) and incubated at 37 ºC overnight. Antimicrobial resistance was evaluated culturing strains in different antibiotic plates with the following concentrations: ampicillin (100 µg/ml), chloramphenicol (34 µg/ml), tetracycline (20 µg/ml), streptomycin (20 µg/ml), and kanamycin (50 µg/ml). Plates were incubated at 37 °C for 18 hours. Among the 30 colonies isolated, Enterobacter cloacae strain amazonensis was selected to conduct the study (ASTOLFI *et al*., 2018).

Figure 1: Igarapé do 40 satellite view in Manaus, Amazonas, Brazil. The arrow indicates the location in which water samples were collected.



Resource: Brazilian Institute of Geography and Statistics.

Plasmid extraction, analysis, and genetic transformation

E.cloacae amazonensis plasmid extraction was performed through the alkaline lysis protocol (BIRNBOIN E DOLY, 1979). DNA concentrations were evaluated using Nanodrop and Qubit following manufacturing guidelines. DNA was analyzed through ethidium bromide-based agarose gel electrophoresis (SAMBROOK, 2001). *E. cloacae amazonensis* plasmid (pEN) was transformed into *Escherichia coli* DH5α through electroporation following the Calvin and Hanawalt protocol (1988).

Mercury resistance assays

Mercury resistance assays were performed using the strains E. cloacae amazonensis, DH5α-pEN harboring the pEN plasmid and DH5α control. Strains were inoculated in selective LB media containing 100 μg/ml ampicillin and incubated at 37 °C for 16 hours with 150 rpm shaking. Pre-culture microbial growth was estimated through optical density measurement at 600 nm. The microbial growth curve was conducted in biological triplicates normalizing strains density in 50 mL of LB media. After 2.5 hours

of growth at 37 °C with 150 rpm shaking, a final concentration of 20 µg/mL of mercury chloride was added to each bacterial culture. Throughout 10 hours of growth, 600 µL of samples were collected every 2.5 hours to measure optical density (OD). Experimental data, average, and standard deviation was analyzed using the GraphPad Prism software.

Heavy metal resistance genes

To investigate the genomic diversity of diverse heavy metal resistant bacteria, we performed comparative genomics of the draft genome sequence of Enterobacter cloacae amazonensis and the complete genome sequence of *Enterobacter hormaechei*, *Enterobacter lignolyticus*, *Enterobacter kobei*, *Enterobacter ludwigii*, *Pseudomonas putida*, *Bacillus subtilis*, and *Cupriavidus metallidurans*. *Cupriavidus metallidurans* and *Pseudomonas putida* are known robust heavy metal resistant and environmental bacteria strains, respectively. Genomic sequences were extracted from the NCBI database with accession numbers presented on Table 1. The Rapid Annotation Subsystem Technology—RAST (AZIZ *et al.*, 2008) server was used to annotate, map to subsystems, and reconstruct metabolic pathways based on its curated prokaryote database. The subsystem Virulence, Disease, and Defense was analyzed in all strains cited on the Table 1 using the custom software GANAS, developed by VIANA *et. al.*, 2020.

Table 1 Species and its GenBank accession number (http://ncbi.nlm.nih. gov/nucleotide).

Species	Strain	Accession Nr
Enterobacter cloacae	*amazonensis*	PZPP00000000
Enterobacter cloacae (reference)	ATCC 13047	NC_014121.1
Enterobacter hormaechei	DSM 16691	NZ_CP017179.1
Enterobacter lignolyticus	SCF1	NC_014618.1
Enterobacter kobei	DSM 13645	NZ_CP017181.1
Enterobacter ludwigii	EN-119	NZ_CP017279.1
Pseudomonas putida	KT2440	AE015451.2
Bacillus subtilis	*subsp. subtilis* 168	NC_000964.3
Cupriavidus metallidurans	CH34	NC_007973.1

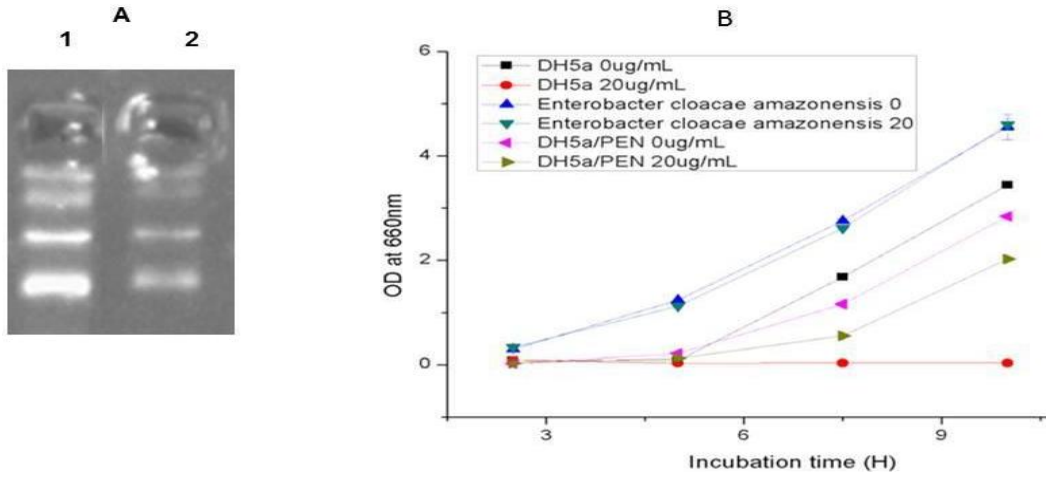
RESULTS AND DISCUSSION

Mercury resistance analysis

Here, we report the heavy metal and antibiotic resistance profile of the Enterobacter cloacae amazonensis strain. E. cloacae amazonensis is resistant to up to 100 µg/mL of mercury chloride in LB solid media, and is multiresistant to the antimicrobials ampicillin, chloramphenicol, tetracycline, streptomycin, and kanamycin, as well as encode for a wide-range of proteins involved in heavy metal metabolism.

The microbial dissemination of resistance genes through horizontal gene transfer by mobile genetic elements is a crucial evolutionary mechanism for heavy metal and antibiotic detoxification in gram-negative bacteria. To investigate the resistance profile encoded by extrachromosomal elements, the pEN plasmid was extracted from *E. cloacae amazonesis* and introduced into *E. coli* DH5α. Transformation was confirmed through plasmid extraction analysis (Fig. 2A1 and 2A2). As a proof-of-concept, growth assays were performed. We observed *E. cloacae amazonensis* and *E. coli* growth curve indicating similar behavior and resistance profile in 20 µg/mL mercury chloride, while the control *E. coli* DH5α did not present resistance. Therefore, the transformation of pEN into *E. coli* enabled the development of resistance mechanisms to mercury (Fig. 2B) and the antimicrobials ampicillin and streptomycin encoded in mobile elements.

Figure 2. Plasmid analysis extracted from *E. coli* and E. cloacae amazonesis through agarose gel electrophoresis. A1) *E. cloacae amazonensis* pEN extracted from *E. coli* after genetic transformation. A2) The native pEN plasmid extracted from *E. cloacae amazonensis*. 2B. Growth curve of *E. cloacae amazonensis* and *E. coli in* 20 µg/mL of mercury chloride.

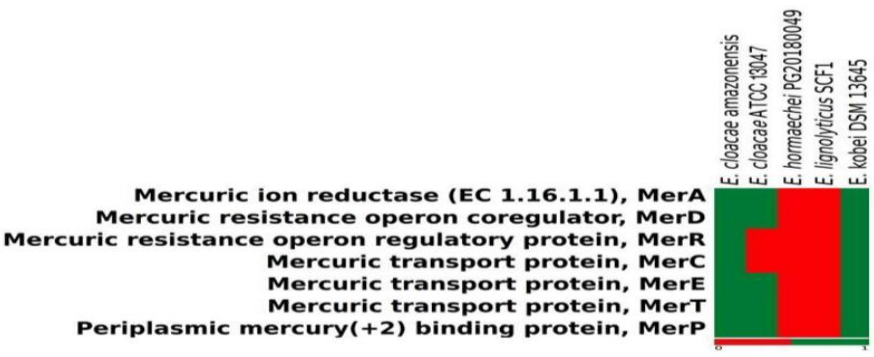


The microbial ability to survive hostile environments is related to the dissemination of mobile elements often encoded in plasmids carrying virulence factors and resistance genes and pathways. This bacterial evolutionary mechanism enables the rapid response and survival of microbes to changes in environmental conditions (REDONDO-SALVO et al., 2020; ZEYAULLAH et al., 2010; VON WINTERSDORFF et al., 2016). Commonly, a correlation of heavy metal and antibiotic resistance genes is found in horizontal gene transfer mechanisms. These genes are colocalized in the chromosome or mobile elements, in which microbes evolved co-selection factors to disseminate resistance to heavy metals and antibiotics in the same plasmid (CHEN *et al.*, 2019; PAL *et al.*, 2015; CHIHOMVU *et al.*, 2015).

Comparative genomics analysis and genomic context

The comparative genomics analysis (Fig. 3) of the mer operon cluster in bacterial species from the *Enterobacter genus.* We observed the presence of the merA, merD, merR, merC, merE, merT e merP genes in the genomes of *E. cloacae amazonensis* and *E. kobei*. The *E. cloacae* ATCC 13047 (NC_014121.1) strain genome does not encode the merR and merC genes, while *E. hormaechei* strain PG20180049 e *E. lignolyticus* SCF1 did not encode to any gene related to mercury metabolism. Interestingly, we detected two copies of the mercury reductase gene (merA), in the E. cloacae amazonensis draft genome, a key enzyme in the mercury reduction pathway (BOYD *et al.*, 2012; LIN *et al.*, 2012**;** BARKAY *et al.*, 2003). We believe that a second copy of the merA gene was an evolutionary duplication event enabling *E. cloacae* amazonensis to better respond to the environmental conditions from which it was isolated.

Figure 3. Comparative analysis of existence of mer operon genes in different bacterial species in the Enterobacter genus.

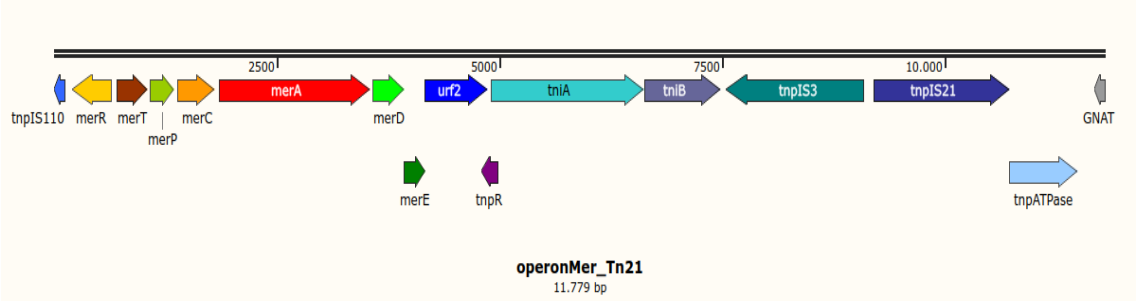


The mer operon genes merRTPCDAB are frequently localized in plasmids and transposons. It can also be observed encoded in the chromosome. Analysis of horizontal transfer patterns of antibiotic and heavy metal resistance genes between bacteria have revealed a large plasmid carrying multiple heavy metal resistance gene clusters in clinical isolates of *E. cloacae* (WU *et al.*, 2018). In *Pseudomonas aeruginosa* EW32, the presence of a conjugative plasmid with co-resistance to tetracycline and copper was detected (MARTINS *et al.*, 2014).

Here, we investigated the contig 24 of the *E. cloacae amazonensis* draft genome. We determined the mercury resistance gene co-localization in the Tn21 transposon family genes encoding transposases. This finding corroborates the mer operon literature localized in the Tn501 and Tn21 transposons, common mobile elements in gram-negative bacteria (LIEBERT *et al.*, 1999). This operon consists of a gene cluster encoding proteins involved in virulence and defense regulation, transport, and enzymatic transformation BETTY *et al.*, 1989; ROSS *et al.*, 1989; NASCIMENTO *et al.*, 2003).

We present here the genomic context in which the *E. cloacae amazonesis* operon mer genes are located. Fig. 4 illustrates the operon mer cluster co-localized with the genes urf2, tnpR (resolvase), tniA and tniB (Tn21 family transposases), tnpIS3, tnpIS21 and tnpIS110 (insertion sequence transposases), and the ATPase and GNAT enzymes, involved in the genetic transposition process.

Figure 4. Genomic context of the mer operon cluster in the contig 24 of the E. cloacae amazonensis draft genome analyzed by the RAST server.



Genes involved in the metabolism of heavy metals and antibiotic resistance

The RAST-server-based annotation of the *E. cloacae* 5.0 Mb draft genome distributed in 69 contigs revealed 579 subsystems and 4.780 coding sequences, among them 95 are RNA coding. We present here the circular visualization of the *E. cloacae* amazonensis genome illustrated in Fig. 5.

Comparative analysis was conducted against the genome of Enterobacter cloacae ATCC 13047 (NC_014121.1), Enterobacter hormaechei strain PG20180049, Enterobacter lignolyticus SCF1, Enterobacter kobei strain DSM 13645, Enterobacter ludwigii strain EN-119, Pseudomonas putida KT2440, Bacillus subtilis WB800N, and Cupriavidus metallidurans. The Virulence, Disease and Defense subsystem was further analyzed using the Análise de Anotação Genômica software—GANAS (VIANA *et. al.*, 2020) generating the Table 2 results, presenting the genes encoding proteins involved in the metabolism of heavy metal and antibiotic resistance.

Figure 5. Circular representation of *E. cloacae amazonensis* chromosome. This circle map was generated using the software Artemis. From inner to outer circle: GC deviation (green and purple), GC content (gray), rRNA (red), tRNA (yellow), protein encoding genes (PEG) colored by subsystems, obtained from RAST webserver annotation of the 69 contigs (alternated in shades of gray).

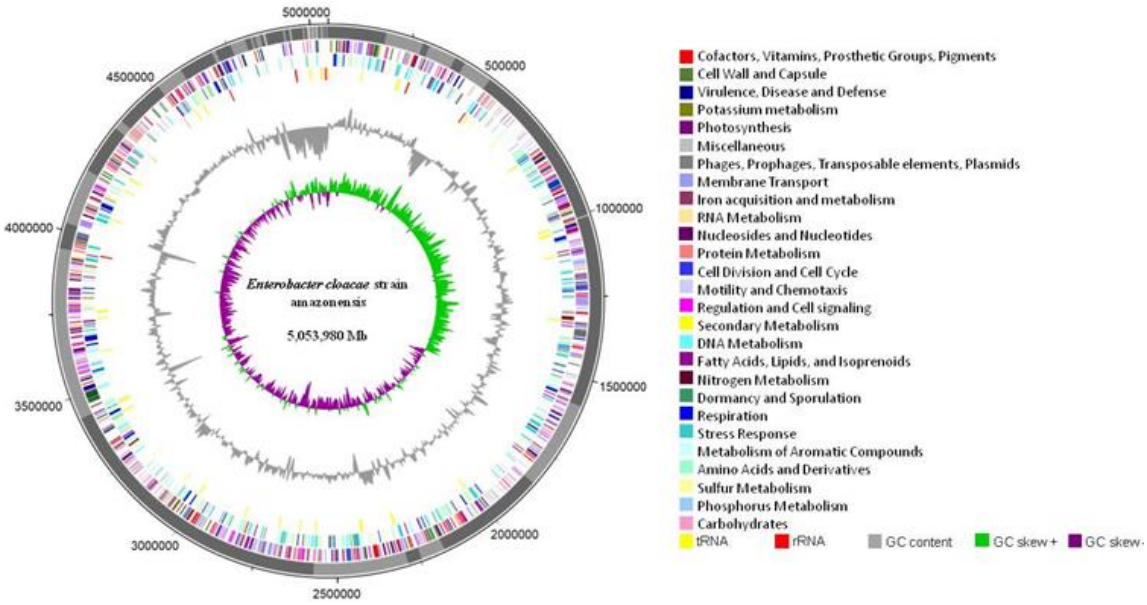


Table 2. Number of annotated genes of the subcategory "*Resistance to antibiotics and toxic compounds*", obtained from RAST web server, for all of the analyzed species. Each cell represents the number of annotated genes of the type of the current row from the species of the corresponding column.

	E. cloacae amazonensis	*E. cloacae* ATCC1307	*E. hormaechei* PG20180049	*E. lignolyticus* SCF1	*E. kobei* DSM 13645	*E. ludwigii* EN-119	*P. putida* KT2440	*B. subtilis* WB800N	*C. metallidurans CH 34*
Copper homeostasis	14	15	5	8	14	7	24	5	11
Copper homeostasis: copper tolerance	8	9	8	8	9	9	2	0	4
Cobalt-zinc-cadmium resistance	23	19	11	14	21	10	30	6	16
Resistance to chromium compounds	2	1	0	1	0	0	1	0	1
Mercuric reductase	2	1	0	1	3	1	0	0	2
Mercury resistance operon	7	5	0	0	12	0	0	0	4
Resistance to fluoroquinolones	4	4	4	4	4	4	4	4	4
Fosfomycin resistance	1	1	1	1	1	1	0	1	0
Beta-lactamase	2	1	3	4	2	4	1	1	1
Arsenic resistance	9	0	2	0	7	5	0	6	0
Lysozyme inhibitors	1	0	0	0	1	1	0	0	0
The mdtABCD multidrug resistance cluster	6	0	6	6	6	8	0	0	0
Aminoglycoside adenylyltransferases	2	0	0	0	0	0	0	0	0
Adaptation to d-cysteine	3	1	3	4	3	3	0	0	0
Multidrug Resistance Efflux Pumps	16	0	21	17	17	23	0	1	0
Multiple Antibiotic Resistance MAR locus	4	0	4	4	4	4	0	0	0
Total	104	57	68	90	104	80	62	24	43

We observed 104 genes involved in heavy metal and antibiotic resistance in the draft genome of *E. cloacae amazonensis* and in the complete genome of *E. kobei*. While harboring the equal gene number, *E. cloacae amazonensis* encodes for genes distributed in all analyzed subsystems, while *E. kobei* does not encode for proteins involved in chromium metabolism. Compared to *E. cloacae amazonensis*, the total number of genes involved in heavy metal metabolism and antibiotic resistance genes was lower in other species. *E. lignolyticus* encodes 90 genes, followed by *E. ludwigii* with 80 genes, *E. hormaechei* with 68 genes, *P. putida* with 62 genes, *E. cloacae* reference with 57 genes, *C. metallidurans* with 43 genes, and finally, *B. subtilis* with 24 genes.

Bacterial adaptive responses and its genomic versatility and plasticity is a well-studied phenomenon involved in the detoxification and mitigation of toxic pollutants. The pangenome analysis of 2489 genomes of the Proteobacteria phylum, among them the *E. cloacae amazonensis*, showed a high abundance of genes related to heavy metals resistance compared to other bacterial phyla (JOHNSON *et al.*, 2019).

Therefore, bacterial genomes bio prospected from copper wastewater presented a high abundance of copper resistance genes (IRAWATI et al., 2021) in the strains isolated from the Indonesian Sukolilo river. *Enterobacter cloacae IrSuk1* and *Enterobacter cloacae IrSuk4a* demonstrated the ability to remove copper through a biosorption method. The multiresistant rhizobacteria *Enterobacter cloacae*, *Enterobacter kobei*, *Bacillus cereus, Rhizobium pusense*, and *Agrobacterium tumefaciens* presents a promising bioremediation potential and demonstrated the ability to remove lead and cadmium, respectively (ABDOLLAHI *et al.*, 2020). In environmental conditions in which metal ions are in excess, it has been observed that *Cupriavidus metallidurans CH34* and *Pseudomonas putida CD2* the synthesis of the protein complex CzcA, a central protein for resistance to cadmium metals, cobalt, and zinc (NIES, D. H., 2003; VON ROZYCKI *et al.*, 2009; HU, N.; MONCHY *et al.*, 2006; JANSSEN *et al.*, 2010; ZHAO, B., 2007).

The high abundance of genes involved in heavy metal metabolism revealed by comparative genomics analysis of the *E. cloacae amazonensis* draft genome, as well as the proof-of-concept assays suggesting mobile element colocalization, suggest plasticity of microbial genetics in light of environmental selective pressure. Here, we believe *E. cloacae amazonesis* is a promising reservoir for genes with heavy metal bioremediation

biosurveillance potential. Genetic engineering and synthetic biology can be a tool to create novel biotechnological applications based on the *E. cloacae amazonensis* bioremediation genetic toolbox.

CONSIDERAÇÕES FINAIS

E. cloacae amazonensis demonstrated an abundant profile of mercury resistance in experimental assays. The prediction of the promising genetic toolbox composed of 104 genes encoding proteins involved in heavy metal metabolism demonstrated the potential of a strain sourced from the Amazon biodiversity.

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