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CRISPR technology and *Cupriavidus metallidurans* as the future of environmental **sustainability**

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Content

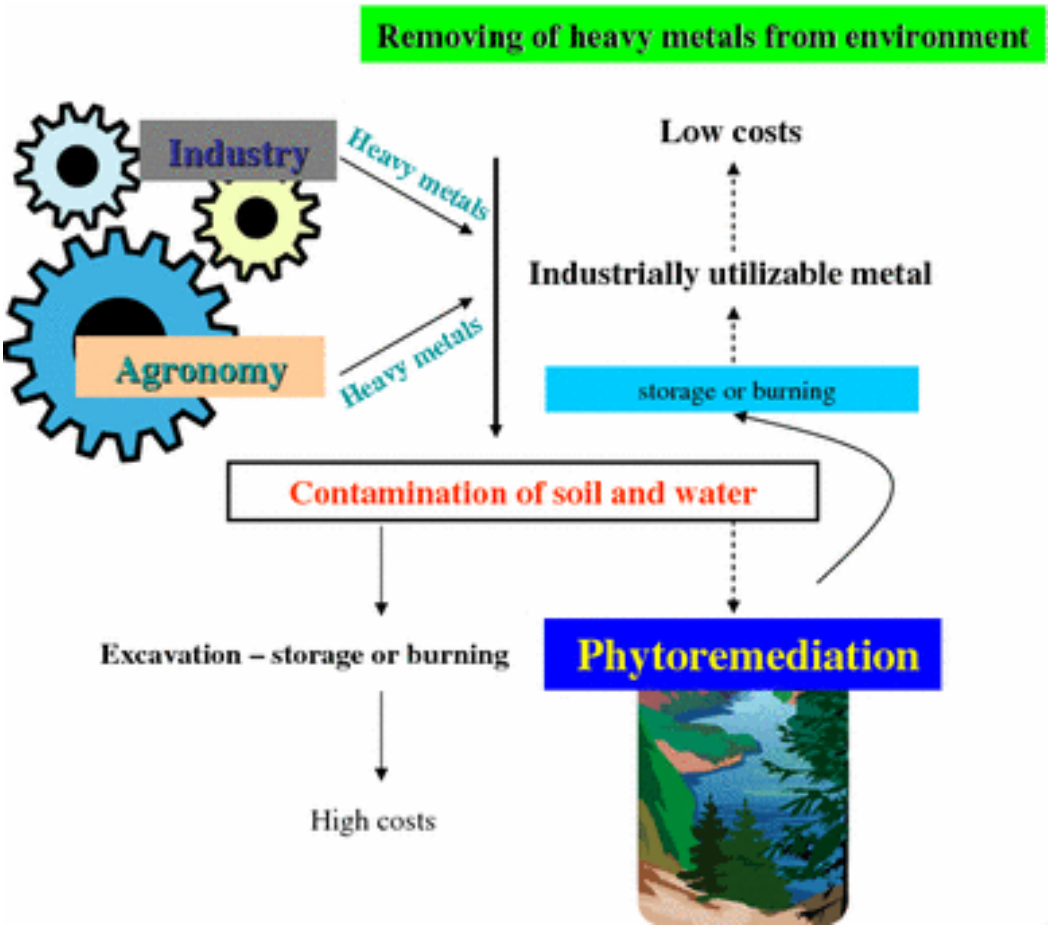
ABSTRACT

This paper discusses the use of CRISPR technology to facilitate and improve the use of C. metallidurans in the industry as a solution to the growing problem of elimination of heavy metals from industrial waste water and from the ecosystems polluted with heavy metals. The properties of C. metallidurans, the possibility of augmentation of the ability to absorb heavy metals in C. metallidurans using genetic modification, and easier implementation of C. metallidurans in the industry by transferring part of the genome of C. metallidurans into E. coli using advanced CRISPR technique are presented. This paper also explains the importance of heavy metal disposal and the potential future of biotechnology use in ecology and beyond.

Introduction:

In the field of genetic modification, a lot of things have been achieved in the last 20 years. By detecting an adaptive immune system called CRISPR (Francisco Mojica and Ruud Jansen, 2002.) the foundations for the successful discovery, ejection and insertion of genes within the genome have been laid. In 2005., Alexander Bolotin studied the bacterium Streptococcus thermophilus (the entire genome has only recently been fully recorded) discovered the Cas9 protein, which is now crucial for genome modification. The next step forward in the study of CRISPR occurred in 2008. when John van der Oost and his team recorded for the first time the important role of gRNA molecules in the CRISPR mechanism. All these findings so far in CRISPR technology, have enabled for Feng Zhang to be able to use CRISPR technology to change the genetic code of human and mouse cells in 2013.. After 2013.. Since 2013., interest in CRISPR has multiplied and the number of scientific papers published on PubMed on CRISPR has grown in just five years (2013. to 2018). from 500 to 4000. Such an increase in interest in CRISPR has enabled faster advances in the technology itself, which is why today CRISPR is the easiest and most efficient method of achieving genetic modification. At the same time as CRISPR technology develops, heavy industry continues to grow. The amount of heavy metal waste has persisted during the 20th century. This creates major problems in ecology and environmental conservation, but also poses a very big problem for human health itself. Elimination of heavy metals from the environment is very complex, expensive and impractical. For this reason, the most common practice for the disposal of heavy metals is the disposal of the waste itself under

the surface layer of the earth. Although such disposal is certainly more economical than the current alternatives, it creates major problems for both the environment and the factories themselves. Although properly performed, such waste disposal always leaves a fairly high probability of penetration into groundwater and can severely contaminate the environment and endanger the surrounding population. In addition to ethical reasons, penetrating heavy metals into the environment, despite proper handling, is punishable by law and factories can face hefty fines for it. The potential solution to these problems was first discovered in 1976. within the wastewater of a metal processing plant in Belgium. It is the Cupriavidus metallidurans bacterium. A bacterium which has adapted to the toxic environment and has developed a unique system of cellular detoxification that protects it from the toxic environment in which it resides. The potential of using this bacteria in solving the problem of industrial waste disposal is the subject of several scientific papers. The biggest obstacles to the use of metallidurans is the inability of this bacteria to survive in certain conditions, which makes it difficult to transport and apply, and the second problem is its rarity. But again, its genome is fully recorded, and the main mechanisms responsible for its ability to accumulate heavy metals have been investigated and the genes responsible for them are isolated, which is why the possibility of creating E. coli and C. metallidurans by using CRISPR technology occurs, which would in fairs allow this biodeployer to be used in the industry and to clean up the environment.



Summary of CRISPR-Cas9 technology mechanisms

“There's already a lot of active research going on using the CRISPR technology to fix diseases like Duchenne muscular dystrophy or cystic fibrosis or Huntington's disease. They're all diseases that have known genetic causes, and we now have the technology that can repair those mutations to provide, we hope, patients with a normal life. '' – Jennifer Doudna

CRISPR-Cas9 is a system used by bacteria to defend against bacteriophage attacks- and has recently been used as a method to precisely cut out the genome at certain points. The system consists of CRISPR-a and Cas9. CRISPR (clustered regularly interspaced short palindromic repeats) represents grouped properly spaced short repetitive palindromic sequences found in the genome within repetitive DNA sequences. Near these reps are cas genes, which encode important enzymes of this system. One of them is Cas9, it's a nuclease – an enzyme that rips nucleic acids (DNA and RNA).

When bacteriophage attacks the bacterium, it inserts its nucleic acid into the bacterial cell, prompting the production of Cas enzymes that cut the virus's nucleic acid into pieces, to incorporate them into its own genome where crispr sequences are located (Fig. 1). In this way, the bacterial cell acquires immunity: namely in the next attack of the same type of virus, the CRISPR sequence, together with viral nucleic acid, is copied into short RNA molecules. These short RNA molecules are first associated with the Cas enzyme, resulting in a complex that recognizes viral nucleic acid, identified on the basis of a match to an already embedded sequence. Then Cas9 cuts the target viral nucleic acid, preventing the virus from using the bacterium for its own replication.

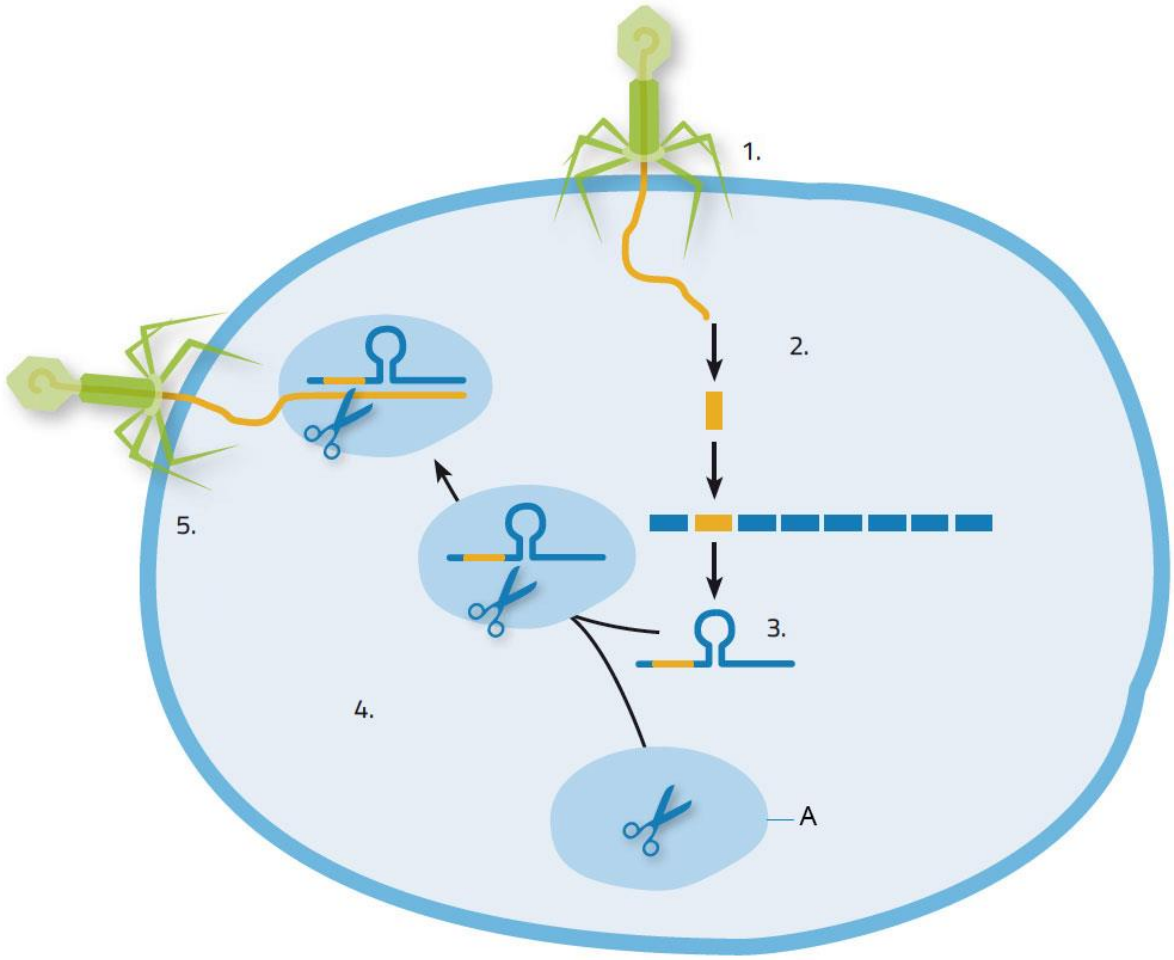


Figure 1: CRISPR-Cas9: the mechanism of bacteria defense. A: Cas9 enzyme

1. Bacteriophage attacks bacterial cell; 2. The nucleic acid of the virus shall be incorporated into the bacterium nucleic acid at the CRISPR site; 3. CRISPR RNA complex is formed; 4. CRISPR RNA binds to the Cas9 enzyme; 5. CRISPR RNA leads the Cas9 enzyme to the virus. It cuts and destroys the viral genome.

How is this bacterial system adapted to develop new gene technology? In 2012., a team of Jennifer Doudna from the University of California Berkeley, USA; and Emmanuelle Charpentier, then from Sweden's Umea University, modified and merged the short RNA molecules into one leading RNA (Figure 2). This allowed one end of the leading RNA to be associated with the Cas9 enzyme, while the sequence at the other end of the chain could make a connection to any target DNA. Such adaptability, made it possible for CRISPR-Cas9 to specifically cut the selected DNA etc. Not long after, Feng Zhang's laboratory at the Massachusetts Institute of Technology, USA, used CRISPR-Cas9 technology to demonstrate its effect in triggering precise gene cutting of human and mouse cells (Cong et al, 2013). In addition, they adjusted the Cas9 enzyme so that it could cut DNA in a little bit different way, simulating a specific mechanism of DNA repair in cells. This means that scientists have

successfully implanted the new DNA sequence into the cut-out site, replacing the original sequence (Fig. 2).

This pioneering step enabled for CRISPR-Cas9 to be transformed from the microbiological environment into an interesting tool of science that allows scientists to rearrange genes for different needs with great precision and ease.

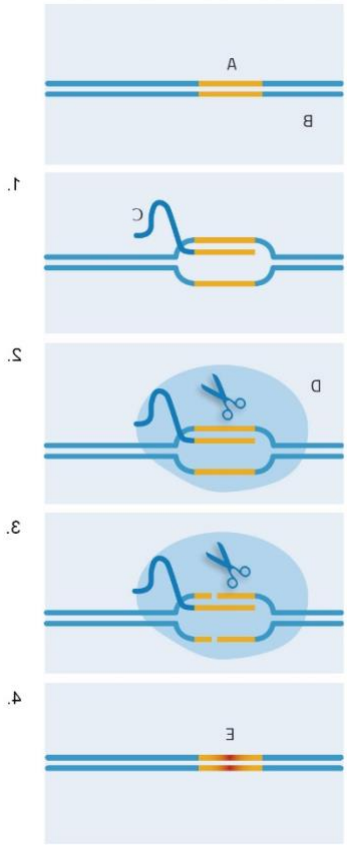
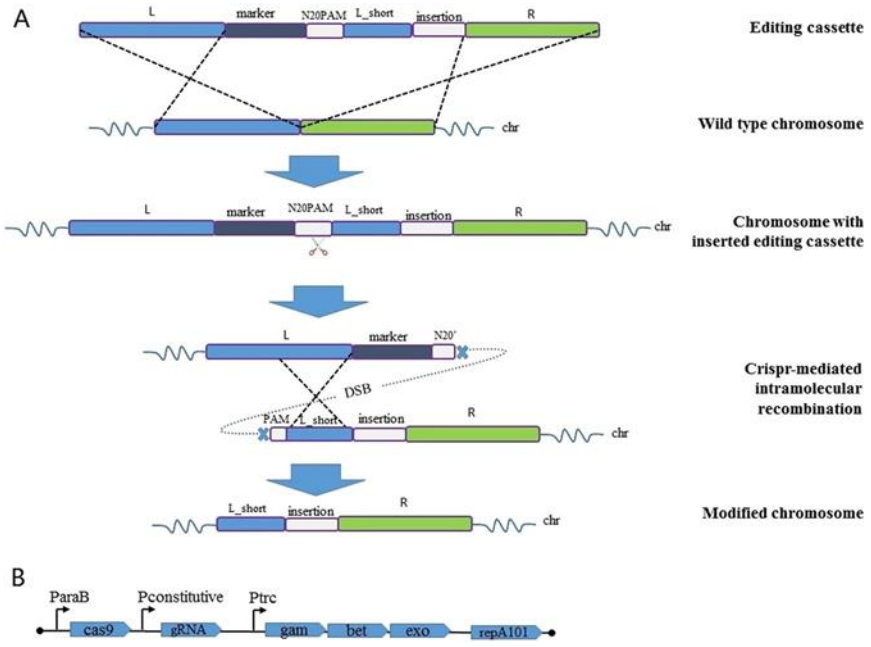


Figure 2: Gene editing
gene editing using
CRISPR-Cas9.
A: Target sequence; B:DNA; C:
Leading RNA; D:Cas9; E:Nova
DNA sequence
1. The leading RNA binds to
target DNA sequence;
2. The Cas9 enzyme is associated with
leading RNA;
3. Cas9 enzyme rips both chains
DNA;
4. Bacterial mechanism
installation of new DNA on the
the location of the chain break,
replacing the original DNA
Sequence.
-Nicola Graf

Analysis of CRISPR capabilities

The CRISPR/Cas9 system is a very powerful and precise means of gene editing. Of course, it has its limits, too. Perhaps the most serious problem that geneticists encounter using CRISPR/Cas9 is the inability of CRISPR to affect all regions of the gene within the genome. There are PAM free areas and areas that show a high degree of tolerance to CRISPR that cannot be edited with conventional CRISPR technology. Because of these problems, 2017. was quite important for the development of CRISPR technology. In 2017, scientists from the Tianjin Institute of Industrial Biotechnology developed a new technology to implement CRISPR called CAGO (CRISPR/Cas9-assistedgRNA-free one-step). The difference between the current applications of CRISPR and CAGO technique is that the CAGO technique does not require the

construction of a specific plasmid for gRNA expression , but uses an universal N20 sequence. Research has shown that this technique can alter much larger gene areas with much greater efficiency. Using CAGO techniques on E. Coli scientists were able to modify an area of 100 kbp with an efficiency greater than 75%. The whole process of gene modification on E. coli using a CAGO technique including gene insertion and confirmation of the modification lasted 1.5 days. The main feature of the CAGO technique is that CAGO simplifies gene editing and also allows for alteration of areas of the genome that cannot otherwise be altered, which together leads to the possibility of modifying larger gene areas with greater efficiency.

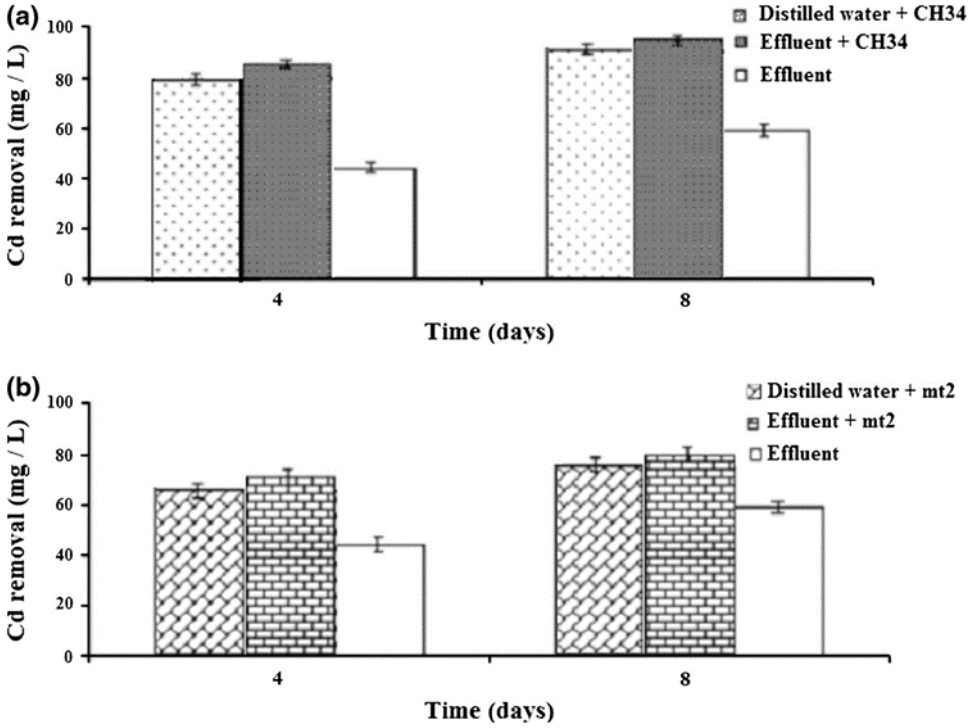


The painting. The scheme presents the CAGO technique of genome modification and pCAGO plasmid.

Cupriavidus metallidurans

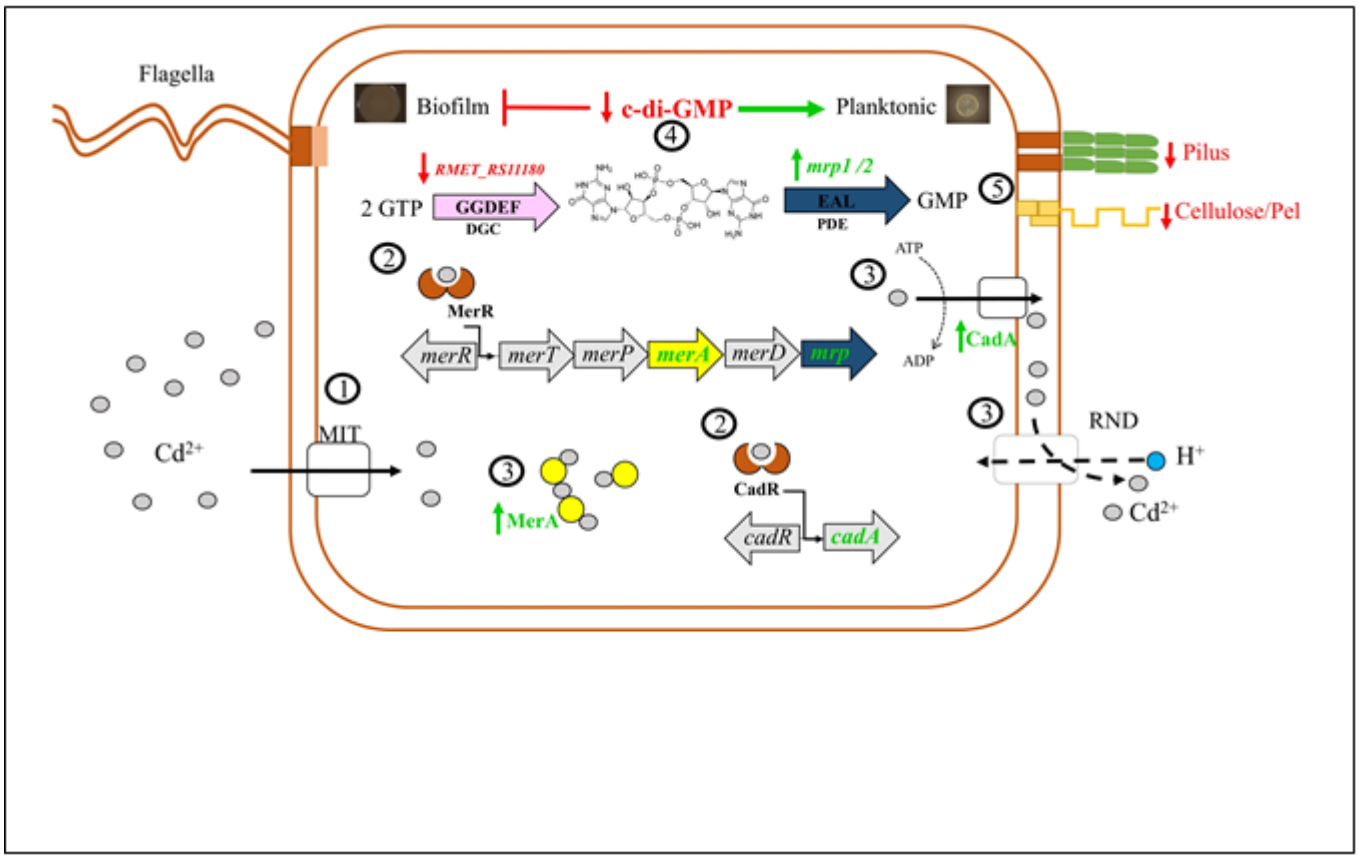
Cupriavidus metallidurans (renamed from Ralstonia metallidurans), is a gram-negative, nonpathogenic bacteria first isolated in a metallurgical plant in Engis, Belgium. The main specificity of this bacterium is high resistance to high concentrations of heavy metals such as Ni(II), Co(II), Hg(II), Ag(II), Cd(II), Cu(II), Pb(II), and Zn(II). All genes related to bacteria's resistance to heavy metals can be found on two large plasmids pMOL28 and pMOL30. PMOL28 plasmid in the 34-kb area contains genes related to resistance to metals Co(II), Cr(VI), Hg(II) and Ni(II), while in the larger plasmid pMOL30, in the area of 132-kb there are genes related to metals Ag(I), Cd(II), Co(II), Cu(II), Hg(II), Pb(II) and Zn(II).. Cupriavidus, due to its resistance to heavy metals, demonstrates the potential to remove toxic heavy metals from the environment, and as a result it is increasingly becoming a topic of scientific papers. In 2014., a survey was conducted which found that C. metallidurans accumulates Cd as follows:

Bacterium	Medium	Time	Cd (mg/L)	Cd total (mg/L)	Percentage
CH34 (C. metallidurans)	Industrial water	4 days	83		
CH34	Industrial water	8 days	93		
CH34	Distilled water	4 days	80	100	80%
CH34	Distilled water	8 days	90	100	90%



(Comparison of Cd accumulation in CH34(metallidurans) and mt2(Pseudomonas putida)**)**

The conclusion of this study confirms the potential of C. metallidurans as a solution to the problem of the management of industrial Cd by means of bioremediation and proves Cupriavidus's advantages before mt2, which except for Cupriavidus demonstrates the best properties for bioremediation of waste heavy metals. In addition to this research, research was conducted investigating the association between metabolic cycles related to heavy metal accumulation and c-di-GMP regulatory pathway. The results of this study indicate that there is no difference in Cd resistance between the biofilm and the plankton form of the bacteria and that the urf 2 gene activated with the presence of Cd inhibits the lowering of c-di-GMP secondary messenger levels. It is assumed that the bacteria favors the plankton form with the Cd present in the system because of easier mobility, which allows it to transition into an area of less toxicity.



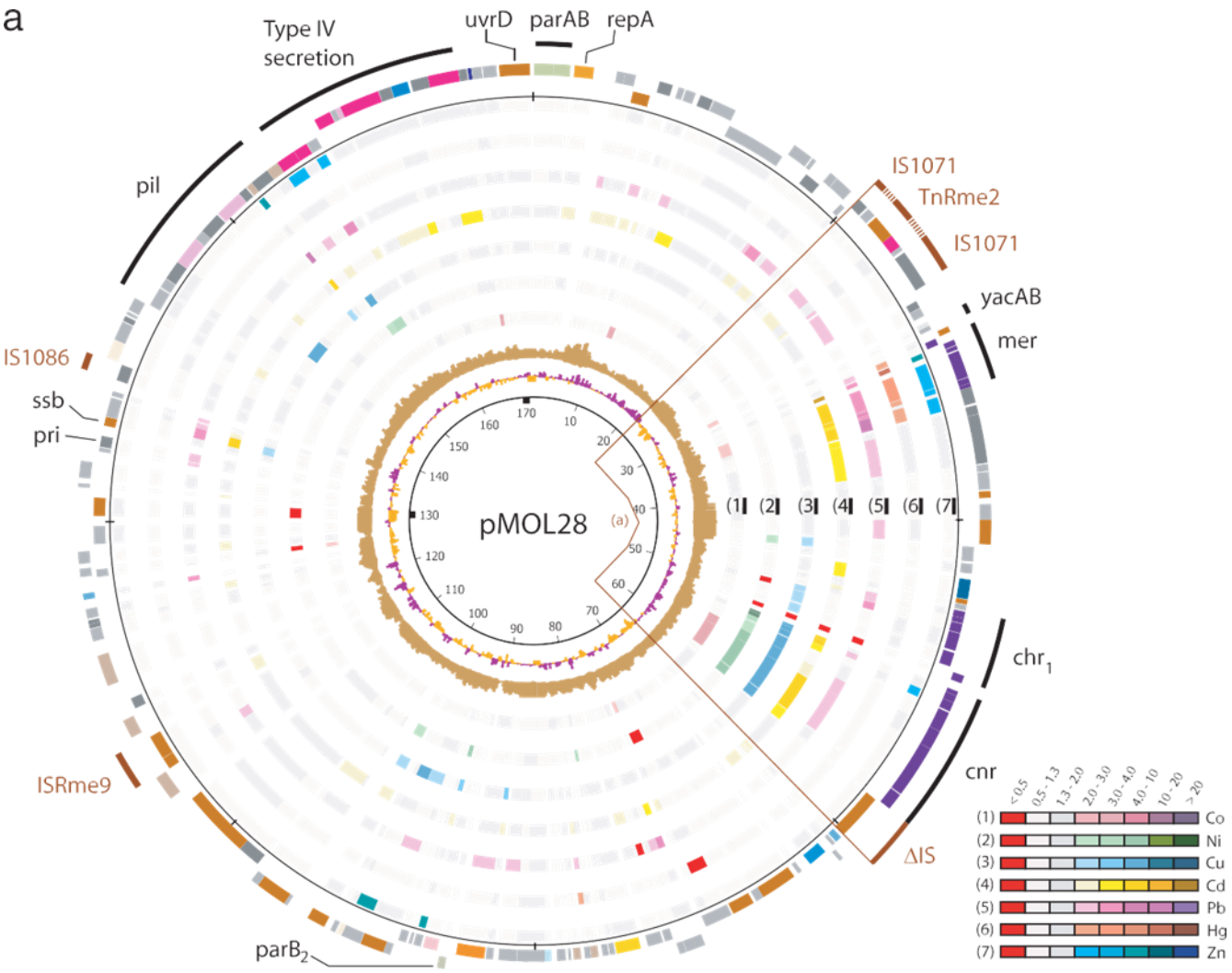
(The figure shows model of C. metallidurans CH34 and c-di-GMP regulatory pathway in the presence of Cd)

1. Cd enters the cell through conveyor on the cell membrane
2. The presence of Cd activates detoxification systems CadA and MerA
3. Cd presence promotes activation of mrp1 and 2 genes and lowers RMET_RS11180 which lowers the level of c-di-GMP secondary messenger

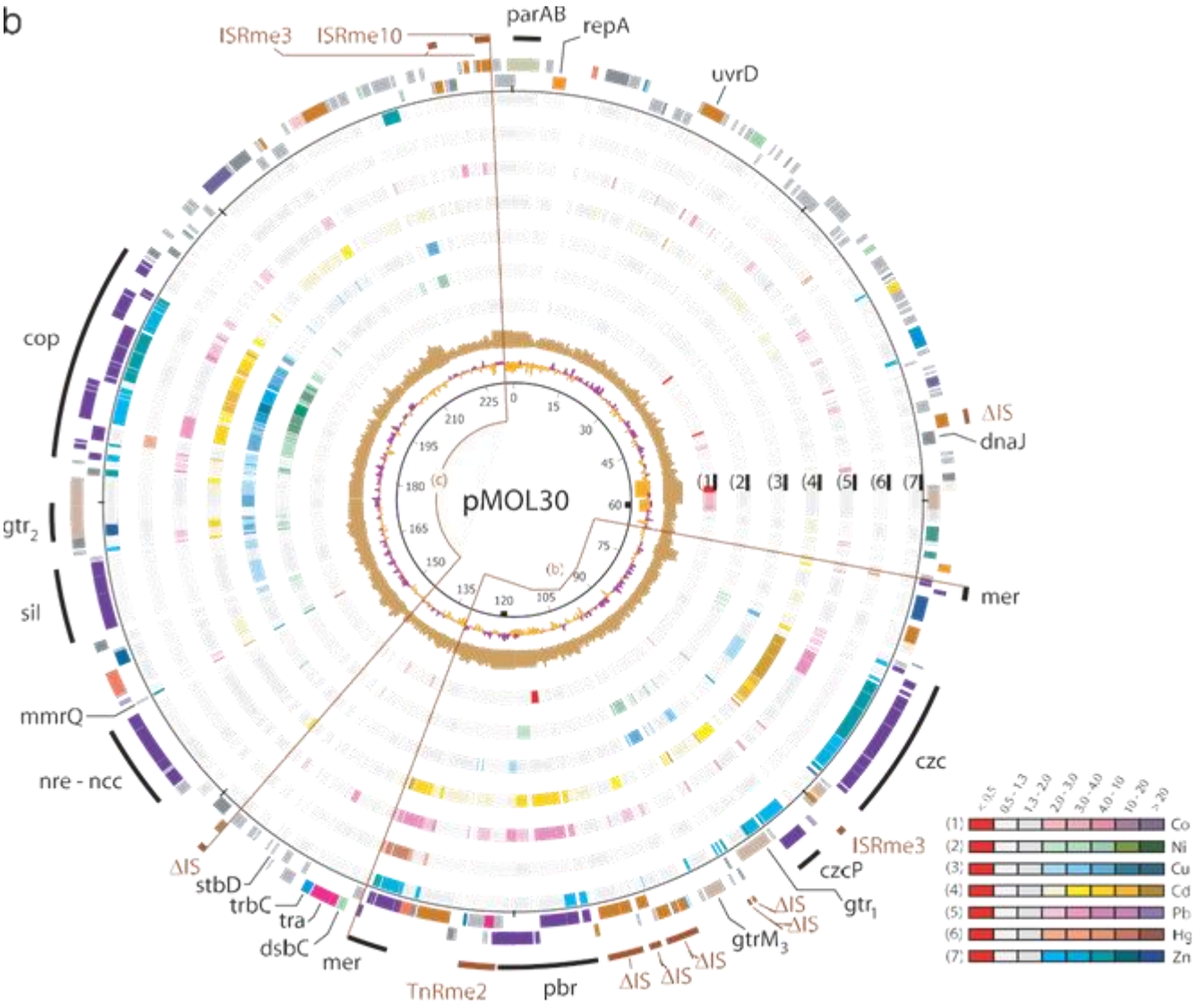
Genetics of Cupriavidus metallidurans

C. metallidurans CH34 has at least 24 groups of genes responsible for resistance and metabolic processes related to heavy metals. All genes C. metallidurans bound to heavy metals are found on two plasmids, pMOL28 and pMOL30. In pMOL28 plasmid they form an area of magnitude 34-kb, and with all indirectly related gene groups the total size is 42-kb. The whole area is on one side surrounded by an IS element from the Tn3 family (next to cnrT

), and on the other side (near merR) with the IS1071 element also from the Tn3 family. In pMOL30 plasmid there are two gene islands related to resistance to heavy metals. The first gene island containing the czc and pbr gene groups is between the Tn4380 transposon and the 3 mer gene. Another island containing cop, sil and nre-ncc group is located between the ISRme10 element and orf-157,158 (very similar to the ISRme10 element). The whole area made up of these two islands is a large 132-kb.



(expression of the pMOL28 plasmid genes in the presence of heavy metals)



(expression of the pMOL30 plasmid genes in the presence of heavy metals)

In 2018., a team of scientists using genetic modification techniques,b inserted a new 54-kb pTP6 plasmid in C. metallidurans CH34 introducing new mer genes thus creating C. metallidurans MSR33. This was done with the intention of creating augmented resistance to Hg in CH34, and the results did not disappoint. Multiple increases in resistance to other toxic compounds have been observed as follows:

Compound	CH_3Hg^+	Hg^{2+}	Cd^{2+}, Co^{2+}	Ni^{2+}
Increase quotient	1,6*	10*	2*	1,2*

Results show that by introducing pTP6 plasmid, C. metallidurans acquires several times augmented resistance to certain heavy metals. For example if we consider Cd, in the research detailed above colony of C. metallidurans absorbed the Cd concentration of 93 mg/L from the industrial water sample within 4 days. If we assume that the concentration of Cd in the water is at least twice as high, this would mean that when considering the boosted absorption capabilities, a strain of metallidurans with pTP6 plasmid may absorb Cd concentration of 186 mg/L over the same period of time, well above the average Cd concentration in contaminated industrial wastewater and in the most affected environments.

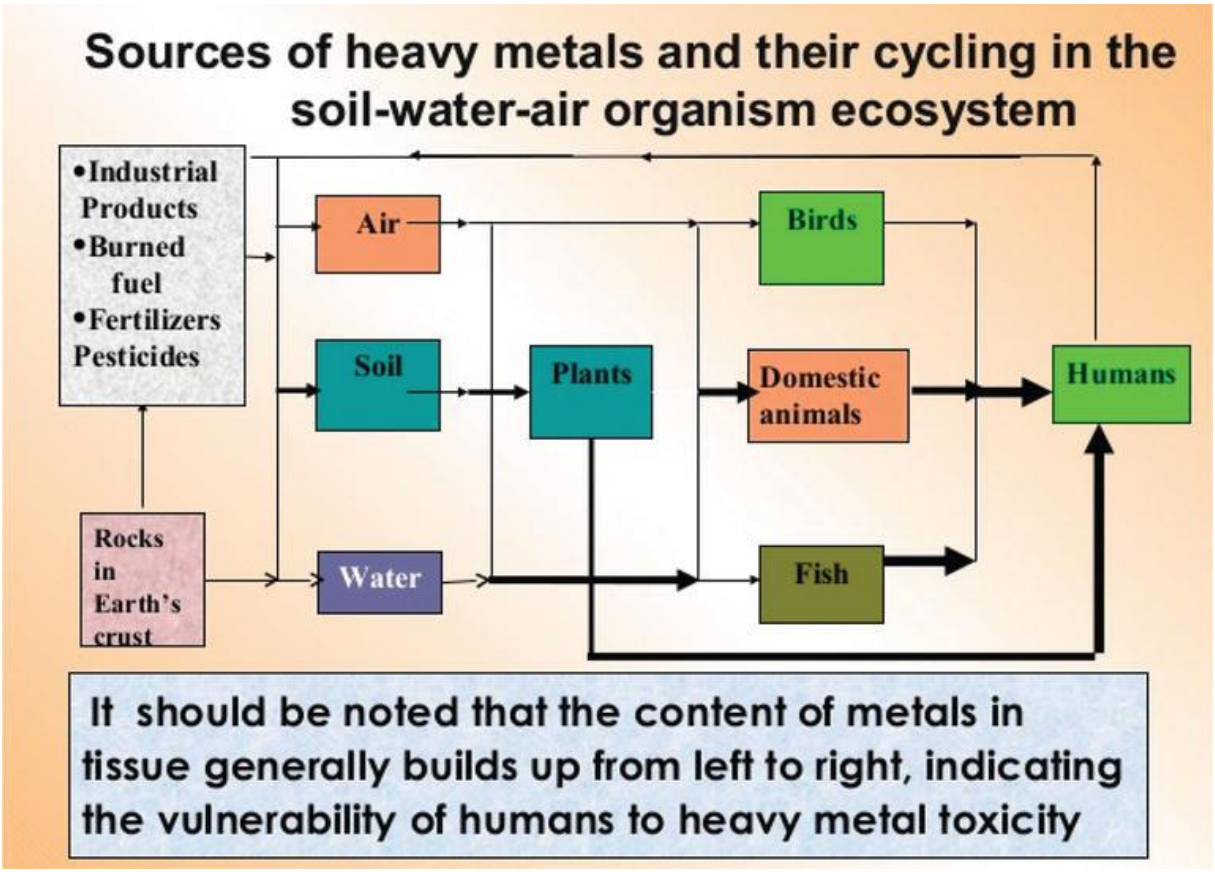
Sediment	**Concentration (mg/kg)**	**Reference**
Korotoa River	1.5	14
Paira River	0.72	16
Buriganga River	3.33	16
Turag River	17.0	15,16,20,40
Bangshi River	0.61	16
Turag River	0.8	16
Karnaphuli River	2.01	14,16

(River Turag is considered one of the most polluted areas by cadmium in Bangladesh)

Toxicity of Cd and other heavy metals in the industry

With the development of the industry since the late 19th century, the use and consumption of heavy metals such as Cd, Co and Hg has been multiplied. With population growth and consequently market growth and ever-growing demand, production is also increasing. Mass production has grown in the last 100 years and thus consequently also increased the environmental pollution by heavy metals . Heavy metals are mostly used in textile and heavy industry, but are present as a by-product in most production mechanisms and therefore appear as the biggest problem of modern industry along with the uncontrolled release of CO_2. Heavy metal ions are highly toxic to mammals and as mass production increases they are entering ecosystems all around the world. Research shows that Cd has destructive effects on the skeleton, liver and kidneys, brain, respiratory, immune and cardiovascular systems. At a

concentration between 1-100 µM, Cd binds to proteins and slows down DNA repair leading to irreversible cell damage. Cr, Hg and Pb are also extremely strong pollutants that mostly affect the brain and cardiovascular systems, which is why WHO regulates their permitted levels in the environment.

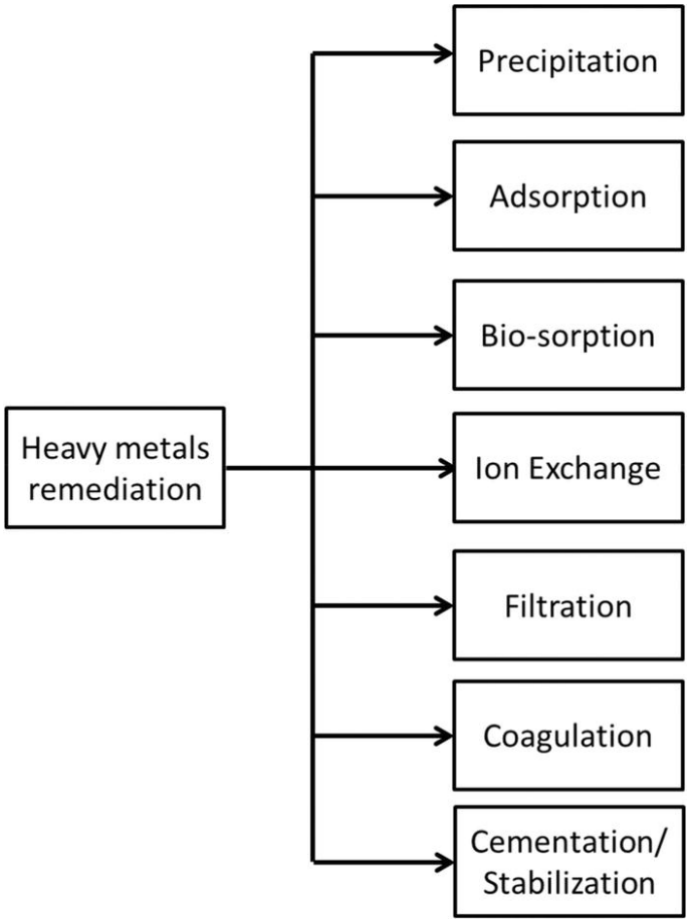


(Cycle of heavy metals passing through the ecosystem)

Application C. metallidurans in ecology

C. metallidurans is applied in two ways in ecology, as a bio-deployer of heavy metal toxic ions and most often as an indicator thereof. Investments in improving the system of C. metallidurans usage as bio-deployer as well as in the possibility of using biotechnology for detoxification of wastewater are being made every year. Why is that? The main advantages of biotechnology over conventional techniques of elimination of heavy metals from the environment are ease of application, thoroughness and relatively low cost. Other forms of elimination of

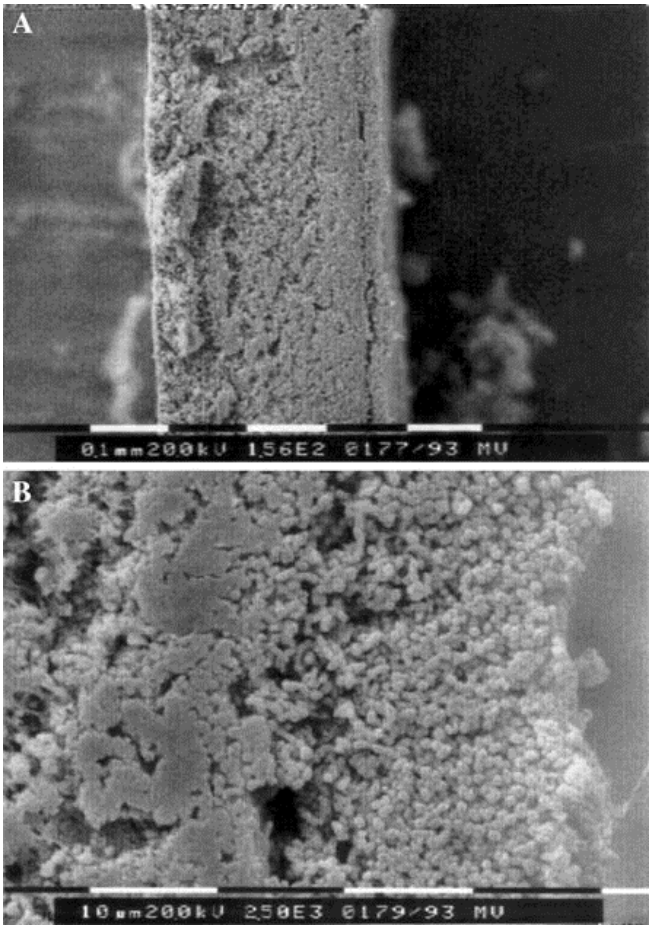
heavy metals include machine removal consisting of several complicated processes in which the contaminated water leaves a significant concentration of toxicants, and chemical removal that is more thorough than machine removal, but is difficult to realize and is significantly more expensive than machine removal.



(Diagram shows different types of elimination of heavy metals from wastewater and contaminated areas)

Previous application and problems

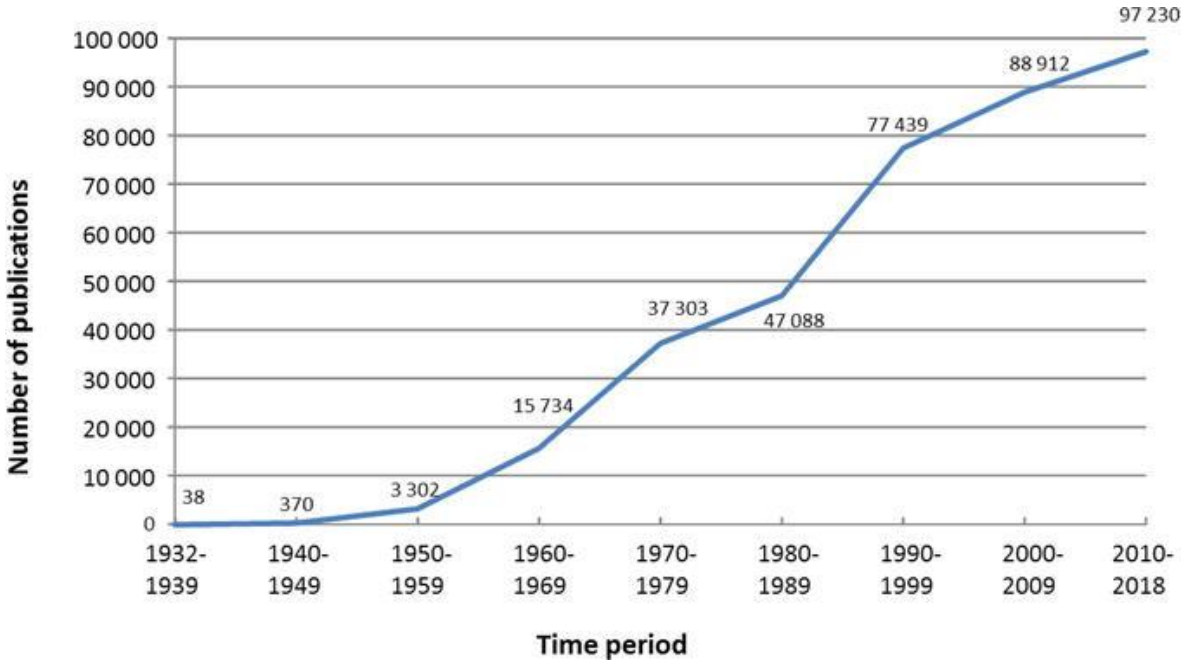
C. metallidurans is a bacterium full of potential for industrial use. Despite many qualities, there are few problems that make Cupriavidus still not conventionally used as a bio-deployer, namely, the fact that using C. metallidurans is a relatively new technology, and when we add that to the rarity of this strain of bacteria and the relatively weak ability of colonies to spread (only 7 days after the introduction of CH34, the colony has been recorded within the system, BUT also remains present in the system over 100 days since its introduction; ref From industrial sites to environmental applications with Cupriavidus metallidurans) we get the full view on downsides of utilizing this technology. Also, although highly resistant to, Cupriavidus is also not resistant to all Ph values, which in certain conditions makes it less usable.



(display of C. metallidurans biofilm colony on the wastewater filter)

Troubleshooting using CRISPR technology

E. coli is a bacterium that creates large colonies in a short period of time and is highly resistant to various environmental factors, making it an ideal carrier for expressing the properties of other organisms by transferring part of the genome from another bacteria to E. Coli. These properties have been experimented with since the earliest beginnings of CRISPR technology, and thus, CRISPR mechanisms have been specifically specialized in genome editing of this bacteria. Also, since E. coli is one of the most common subjects of genetic modification, a number of strains of E. coli have been made. Some strains are genetically modified so they can survive in different extreme conditions. All the reasons described are showing us that E. coli truly is the ideal carrier of the usable genetic properties of other bacteria. Using E. coli as carriers of gene group of C. metallidurans responsible for mechanisms related to heavy metals ensures easier and faster absorption of heavy metals from the environment and enables use in almost all environments (different genetically perfected E. coli strains can be used for extreme environments (e.g. E. coli O157:H7 for acidic environments). Also, by inserting additional plasmid pTP6 (in addition to plasmids pMOL28 and pMOL30), the capability of absorption properties multiplies.



(Number of published studies with the theme regarding E. coli through time (1932-2018)

Implementation theory

Technology of using E. coli augmented by genetic determinants of C. metallidurans can be used in two ways, by growing a colony on the walls of a filter set to the flow of liquid water and by releasing augmented non-pathogenic bacteria into the biosphere of stagnant waters. The recent crisis regarding pollution of Neretva river by heavy metals (3.2020.) may serve as an experiment for exploring the usefulness of the supposed model of technology in vivo .

(04.2019, 1st degree alert due to pollution of the Krka River)

Economy of future technology and announcement of the future of genetics

CRISPR is a fairly new technology, but it's already showing incredible results. It gives biologists the ability to do more than ever before, not only study but reproduce certain genetic characteristics. Even before the advent of CRISPR technology, there was genetic modification, but CRISPR provides precision sufficient to make much bigger changes to the genome than ever before. Through IT terminology it can be shown in a simplified way that genes are parts of software, CRSIPR copy-paste function, and E. coli is the basic model of a programmable robot. Of course, genes create themselves more advanced special pieces of hardware and E. coli only serves as a casing. In the future, it can certainly be expected that biotechnology will gain significance, probably as fast as IT has in the time period from the 80s to the present. Over many years, evolution has made different adaptations to the various problems that living organisms have encountered throughout the history of the Earth. CRISPR technology enabled a better understanding of the mechanisms of genetics and for the first time in human history it also allowed a direct impact on the genetic makeup of living beings. How can we use that? If the parallel with informatics is drawn, it can be set like this:

1. Nature has developed a number of programs (organisms, bacteria) whose functions (biological possibilities) are very diverse
2. Their function is determined by nature to assist in the performance of the main task that nature has given (survival, conservation of species)
3. Programs surviving in difficult conditions are less frequent, but have more specific and complex functions
4. Most of the complex properties of these functions (mechanisms, adjustments) cannot be reproduced by electronics and machinery
5. CRISPR gives the ability to combine multi-organism functions to create a tailored program with all the necessary functions to perform a task much more complex than the original goal (survival)

Electronics have its limitations, and the main limitation for performing complex tasks is always hardware. On the example of heavy metals, software can not in itself eliminate heavy metals from the environment, and hardware that is so complex to try to do that, has its limitations and is quite expensive. It is precisely for these and similar reasons that it can be assumed biotechnology will experience drastic growth in the industrial use. By perfecting genetics, and by pushing genetics into a wider range of industry applications, many of the tasks considered extremely complex and basically impossible to finish will become quite simple and economically sustainable. Biology is, in its broader sense, really the industry of the future. The next step that comes after automation. The elusive quintumal sector is not a

complete automation and itinerant society, but a nature-given programming language, i.e. biotechnology.

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