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(54) **THE REMOVAL OF ARSENIC USING A DISSIMILATORY ARSENIC REDUCTASE**
DIE VERWENDUNG VON ARSEN REDUCTASE ZUR ENTFERNUNG VON ARSEN
L'ÉLIMINATION D'ARSENIC A L'AIDE DE L'ARSENIC REDUCTASE

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Description

TECHNICAL FIELD

[0001] The object of the invention is the plasmid pSheB, particularly a plasmid comprising a fragment of pSheB comprising the *arr* module, and strains comprising such a plasmid, particularly *Shewanella* sp. O23S strain, which are capable of removing arsenic from mineral resources, raw materials industry waste and soils, in particular, post-mining (eg. soils) under anaerobic conditions, by dissolution of minerals and reduction of arsenates to arsenites. The object of the invention is also the method and the use of such bacterial strains or a composition comprising them, for the selective removal of arsenic from mineral resources, raw materials industry waste or the soil.

BACKGROUND ART

[0002] Arsenic is an element that very often co-occurs in copper minerals and constitutes their specific impurity. In pyrometallurgical processes of roasting and smelting of copper concentrates, volatile arsenic compounds are released into the atmosphere, which, due to the toxicity of these compounds, constitutes a major threat to the environment. During the smelting process, most of the arsenic is removed as a volatile compound As_4O_6 at concentrations up to 0.5 mg/l, while only 0.04%-0.06% is removed in a solid, stable form with slags [Piret, 1999]. Apart from the volatile arsenic compounds, high concentrations of arsenic are also found in dusts. For this reason, it is very important from both an economic and an environmental point of view, to develop an effective method of controlled removal of arsenic from copper deposits and the products of their processing.

[0003] In order for the removal of arsenic from copper minerals to bring the expected economic and environmental benefits, this process must be conducted at the early stages of the copper deposits processing, such as flotation. The traditional copper flotation systems are insufficient and inadequate for separation and division of sulphide minerals containing arsenic (e.g. enargite Cu_3AsS_4 or tennantite $(\text{Cu},\text{Fe})_{12}\text{As}_4\text{S}_{13}$) from copper sulphides not containing arsenic, present in the ores. An aid to the conventional flotation methods are the methods proposed in recent years. One of the methods relates to the selective oxidation of sulphides, based on the electrochemical properties of the separated compounds [Fornasiero et al., 2001]. Another method of selective flotation utilizes the differences in the flotation pulp potentials [Guo and Yen, 2005]. Using the separation method based on the difference in the pulp's potentials, minerals containing arsenic, e.g. enargite (Cu_3AsS_4), can be separated from copper sulphides not containing arsenic. As a result of these processes, two fractions of concentrates are produced: (i) with a low arsenic content, and (ii) with a high arsenic content. The former can be used in pyrometallurgical processes, whereas the latter fraction of concentrates, containing copper minerals contaminated with arsenic, still requires adequate treatment [Senior et al., 2006].

[0004] One of the ways that can help to solve the problem of removal of arsenic from copper minerals, is the application of biohydrometallurgical methods, using microorganisms to recover metals from minerals and deposits. The use of microorganisms for the extraction of copper or gold from their ores and concentrates, is a well-known process, and is often described in the literature [Xia, L. et al., 2010; Xia, L. et al., 2009; Olson et al., 2003; Rawlings and Johnson, 2007]. Most of the biohydrometallurgical processes are based on the processes of oxidation of minerals, and lead to (i) an increase in the accessibility to chemical solvents (biooxidation) or (ii) their direct dissolution (bioleaching) [Rawlings and Johnson, 2007]. Unfortunately, these methods are non-specific, because they are based on the oxidation of sulfur and/or iron from minerals, and are associated with the release of all the metals associated with this type of minerals. A further limitation of the traditional biohydrometallurgical methods is the use of acidophilic bacteria in leaching from neutral or slightly alkaline deposits, which is often inefficient, and sometimes even impossible, due to the need for the use of considerable amounts of sulfuric acid to acidify the deposits. In the literature microorganisms are described, mainly chemolithoautotrophic, sulfur oxidizing bacteria, belonging to the genus: *Thiobacillus*, *Halothiobacillus*, *Thiomonas*, and iron oxidizing bacteria, such as: *Galionella feruginea* or *Leptothrix ochracea*, *Thiothrix* and *Beggiatoa*, which can be used in bioleaching processes at neutral pH, but are very difficult to cultivate and are still poorly understood. Furthermore, bioleaching with the use of these microorganisms is time-consuming and these processes are carried out as long as several months [Skłodowska and Matlakowska, 2007]. A confirmation of the lack of suitable microorganisms, capable of recovering metals under neutral or slightly alkaline conditions is the current situation in the mining market. Currently, there are no known and commercially available biotechnological methods of removing precious metals, occurring in the form of sulphides, under the conditions of neutral or slightly alkaline deposits.

[0005] An alternative to the oxidation processes are selective bioreduction processes, in which the selected elements, associated with the metabolic activity of microorganisms, are released. Although there are several examples of application of the bioreduction processes, methods for removing arsenic from copper minerals using microbial reduction are unknown. Many strains of bacteria that dissimilatory reduce arsenates have been identified, but the use of most of them is limited to the transformation of soluble arsenic compounds [Newman et al., 1998] or secondary arsenic minerals, resulting from iron compounds [Zobrist et al., 2000]. Strains capable of removing arsenic from copper concentrates and flotation tailings

have also been described [Mantur et al., 2011], but the dissimilatory arsenate reduction process, carried out by the aforementioned strains, is not fully balanced and part of the arsenic may be removed out of the cells in the form of volatile, toxic arsenic compounds (unpublished data). Apart from that, the strains described by Mantur et al., 2011, simultaneously release copper and arsenic from minerals, thus lowering the value of the obtained copper concentrate.

DISCLOSURE OF THE INVENTION

[0006] In the light of the described state of the art, the aim of the present invention is to overcome the indicated inconveniences and to provide a plasmid comprising genetic information ensuring the capability of dissimilatory arsenate reduction and selective removal of arsenic, particularly from copper deposits. The aim of the invention is to provide novel bacterial strains comprising such a plasmid, compositions comprising them and uses thereof, and the methods for the selective removal of arsenic using such strains. Furthermore, it is desirable for such microorganisms, possessing the capability of arsenate reduction, not to produce volatile, and, at the same time, toxic arsenic compounds. The *Shewanella* sp. O23S strain, that has been isolated from microbial mats from a gold mine in Zloty Stok, possesses such properties [Drewniak, 2009]. The *Shewanella* sp. O23S strain has been deposited on 24 July 2012 in the IAFB Collection of Industrial Microorganisms Institute of Agricultural and Food Biotechnology in Warsaw, Poland under the deposit number KKP 2045p. This strain is capable of anaerobic growth using arsenates as the final electron acceptor and can mobilize arsenic from rocks from the gold mine in Zloty Stok [Drewniak et al., 2010]. It was unexpectedly found, that these properties are ensured by the plasmid pSheB, isolated from *Shewanella* sp. O23S, the sequence of which has been shown in SEQ ID NO: 1, particularly its region comprising the fragment from 63978 to 72599, which encodes, among others, dissimilatory arsenate reductase, determining these properties.

[0007] Bacterial strains comprising a plasmid including the fragment from 63978 to 72599 of SEQ ID NO: 1, plasmid pSheB, particularly the *Shewanella* sp. O23S strain (deposited as KKP 2045p), are capable of dissimilatory arsenate reduction and selective removal of arsenic, particularly from mineral resources, raw materials industry waste and from the soil, particularly preferable is that they are capable of dissimilatory arsenate reduction and selective removal of arsenic from copper deposits, preferably under neutral or slightly alkaline conditions. The bacterial strains comprising the plasmid comprising the fragment from 63978 to 72599 of SEQ ID NO: 1, pSheB, particularly the *Shewanella* sp. O23S strain (deposited as KKP 2045p) do not produce toxic, volatile arsenic compounds and stably persist in the environment.

[0008] The invention therefore relates to the isolated plasmid comprising the fragment of the nucleotide sequence from 63978 to 72599 of the plasmid pSheB, having the sequence shown in SEQ ID NO: 1.

[0009] The invention also relates to the plasmid pSheB shown in SEQ ID NO: 1.

[0010] The term "functional derivative of the plasmid" or "functional derivative of the sequence" comprises plasmids/sequences having a nucleotide sequence coding for open reading frames, which encode products comprising an amino-acid or a nucleotide sequence identical or highly homologous to the sequences coded by the indicated sequences, wherein the coding sequences or other sequences of the plasmid/sequence have been modified e.g. by substitution, replacement, deletion or insertion, such that it does not essentially alter the activity of the products of these open reading frames, and enables the maintenance of functional features carried by such plasmid/sequence. The indicated sequence will therefore be the region comprising the fragment from 63978 to 72599 of SEQ ID NO: 1, equally preferably it will be the sequence of the plasmid pSheB shown in SEQ ID NO: 1. A highly homologous sequence means that the sequence is homologous, preferably identical in at least 70%, preferably 80%, more preferably 90%, most preferably, in at least 95%. The term "functional derivative of the plasmid" means, therefore, plasmids having a nucleotide sequence coding for open reading frames, encoding products comprising an amino-acid or a nucleotide sequence identical or highly homologous to the sequences coded by the fragment from 63978 to 72599 of SEQ ID NO: 1 and/or to the sequence of the plasmid pSheB shown in SEQ ID NO: 1, wherein the coding sequences or other plasmid sequences have been modified e.g. by substitution, replacement, deletion or insertion, such that it does not essentially alter the activity of the products of these open reading frames, and enables the maintenance of functional features carried by such a plasmid.

[0011] The essence of the present invention is thus based on an unexpected finding, that it is possible to use a strain containing the plasmid comprising the nucleotide fragment from 63978 to 72599 of SEQ ID NO: 1, pSheB shown in SEQ ID NO: 1, particularly the *Shewanella* sp. O23S strain, deposited as KKP 2045p, for the selective removal of arsenic from mineral resources, raw materials industry waste and soils, preferably under neutral or slightly alkaline conditions. It was unexpectedly found, that the strain comprising the plasmid pSheB, shown in SEQ ID NO: 1, particularly the *Shewanella* sp. O23S strain, is: capable of (i) growth in mineral media containing bituminous black shales, flotation tailings, and post-mining soils containing arsenic, (ii) selective release of arsenic from copper minerals containing arsenic, (iii) tolerating the toxic effects of heavy metals released as a result of dissolution of minerals, is (iv) lacking the ability to produce volatile arsenic compounds, is (v) lacking the ability to mobilize copper from mineral resources. The present invention, therefore, also relates to the *Shewanella* sp. O23S strain, comprising the plasmid pSheB, deposited under the number KKP2045p in the IAFB Collection of Industrial Microorganisms Institute of Agricultural and Food Biotechnology

in Warsaw.

[0012] The invention also relates to a composition comprising the isolated plasmid according to the invention and/or a bacterial strain according to the invention or combinations thereof.

[0013] The invention also relates to use of a bacterial strain according to the invention, a composition according to the invention, for the selective removal of arsenic from mineral resources, raw materials industry waste or the soil.

[0014] Particularly preferred is the use of the bacterial strain, *Shewanella* sp. O23S, comprising the natural plasmid pSheB, carrying: (i) all the genes necessary for dissimilatory arsenate reduction, (ii) arsenite and arsenate resistance genes, and (iii) genes coding for the replication-stabilization system for selective removal of arsenic from mineral resources, raw materials industry waste and post-mining soils. The complete sequence of the plasmid pSheB of *Shewanella* sp. O23S has been shown in SEQ ID NO: 1.

[0015] The presented solutions according to the invention enable the removal of arsenic from mineral resources, raw materials waste, and post-mining soils, preferably under neutral or slightly alkaline conditions, preferably at a pH in the range of about 6 to about 8, using a strain comprising the plasmid pSheB, more preferably *Shewanella* sp. O23S, without the need for acidification of the "environment" and without the risk of releasing toxic, volatile arsenic compounds. By the invention, it is possible to selectively remove arsenic without the undesirable release of the target metals, e.g. copper or gold.

[0016] The invention therefore relates to the method for selective removal of arsenic from mineral resources, raw materials industry waste, or the soil, in which the dissimilatory arsenate reduction step is carried out using a bacterial strain according to the invention and/or a composition according to the invention. Preferably, the dissimilatory arsenate reduction step is carried out under neutral or slightly alkaline conditions. Preferably, the mineral resources are copper deposits.

[0017] The invention also relates to the method for selective removal of arsenic from various mineral resources, raw materials industry, or soils, in which the removal of arsenic is carried out by dissimilatory arsenate reduction, using a bacterial strain according to the invention, comprising the plasmid pSheB having the sequence shown in SEQ ID NO: 1, more preferably the *Shewanella* sp. O23S strain, deposited as KKP2045p, which method comprises the following steps:

a) preparing of the mineral resources, wastes or soils and mixing with an appropriate culture medium to enable the cultivation of the strain,

b) adding an inoculum of said strain and carrying out the culture under conditions enabling the growth of said strain and the conduction of dissimilatory arsenate reduction.

[0018] It is preferable when step b) is followed by step c) selectively removing arsenic, released from the solutions obtained in step b). Such removal of arsenic from the solutions (culture fluids), will preferably be carried out using the already developed methods, e.g. by flotation, preferably by selective precipitation of arsenites with sulphides, as a consequence of which a stable, water-insoluble compound, arsenic sulphide As_2S_3 is formed [Robins, 1985].

[0019] In the preferred method, step a) is carried out by: (i) shredding and fractionation of mineral resources, wastes and soils, preferably to the fraction of 125-250 μ m (having the average particle size), due to the highest performance obtainable in this range of particle sizes. Equally preferred is (ii) the preparation of an appropriate culture medium R1-R2 with additives of suitable substrates, among which are: sodium lactate as the source of carbon and energy, yeast extract as an additional source of carbon and vitamins, Tuovinen's mineral salts (*Tuovinen salts (Tuovinen and Kelly, 1974)) as the source of microelements. Furthermore, in the preferred method, the medium does not contain NO_3^- and Fe^{3+} .

[0020] It is equally preferred if in step b) the culture is carried out under conditions of an appropriate, anaerobic atmosphere, which is obtained by conducting the culture with flushing of the medium with a mixture of gases $N_2:CO_2$, preferably in a ratio 4:1. The flushing of the medium with the mixture of gases $N_2:CO_2$, preferably in a ratio 4:1, can be equally used already in step a) of the method of dissimilatory arsenate reduction.

[0021] In the disclosed preferred method, in order to obtain the highest performance for the *Shewanella* sp. O23S strain, step b) is carried out at a temperature in the range from 15°C to 30°C, preferably about 22°C with shaking (160 rpm), for at least 21 days. It is preferred if the density of the culture at the beginning of the process is at least about 10^6 cells/ml. In the preferred method, the inoculum of the *Shewanella* sp. O23S strain is flushed several times with saline solution or is passaged several times to R1-R2 medium, enriched with sodium arsenate.

BRIEF DESCRIPTION OF DRAWINGS

[0022] For a better understanding of the invention, it has been illustrated in the examples of embodiments and in the accompanying figures, in which:

Fig. 1. Shows the genetic organization of the plasmid pSheB. In the diagram, different modules of the plasmid's structure and phenotypic regions have been described: REP/STA - replication-stabilization module, TA - toxin/an-

titoxin module, TRA - conjugation module, SIDERO-siderophore production module, STA - additional stabilization module, ARS - arsenic metabolism module, and SOS - SOS repair system module.

Fig. 2. Shows a comparison of the capability of dissimilatory arsenate reduction by a wild-type strain (wt) *Shewanella* sp. O23S deposited as KKP2045p (harbouring the plasmid pSheB) and its derivative, deprived of the plasmid pSheB. In order to compare the abilities of the investigated strains to reduce arsenates to arsenites, anaerobic cultures were carried out in minimal R1-R2 medium containing 2.5 mM (187.5 ppm) of sodium arsenate. As(V) and As(III) content in culture fluids collected from the cultures every 24 hours is shown on the graph. • - indicates the concentration of As(V), ■ - indicates the concentration of As(III), a solid line indicates the kinetics of As(V) reduction, carried out by the wild-type strain, while a dashed line - by the derivative, deprived of the plasmid pSheB.

Fig. 3. Shows a graph illustrating the efficiency of the process of arsenic removal from mineral resources by the *Shewanella* sp. O23S. strain for A) flotation tailings, B) bituminous shales. Sterile R1-R2 media, enriched with the respective mineral resources: (A) flotation tailings ("middlings") and (B) bituminous shales ("shales"), were used as the control samples.

Fig. 4. Shows a graph illustrating the efficiency of the process of copper removal from mineral resources by the *Shewanella* sp. O23S. strain for flotation tailings ("middlings") and bituminous shales ("shales"). Sterile R1-R2 media, enriched with the respective mineral resources: flotation tailings ("middlings") and bituminous shales ("shales"), were used as the control samples.

Fig. 5. Shows a graph illustrating the efficiency of the process of arsenic removal from the soil contaminated with arsenic by the *Shewanella* sp. O23S strain. In order to confirm the ability of the investigated strain to remove arsenic from the soil, a soil sample originating from the Zloty Potok area was used, and an anaerobic culture in minimal R1-R2 medium was carried out. Arsenic content in culture fluids collected from the cultures every 24 hours is shown on the graph. Sterile R1-R2 media with the addition of the soil were used as the controls.

Fig. 6. Shows a graph illustrating the efficiency of arsenic removal by the *Shewanella* sp. O23S strain in a medium containing 2.5mM (187.5ppm) sodium arsenate and 5mM sodium thiosulfate. Sterile R1-R2 media with sodium arsenate and sodium thiosulfate were used as the controls.

Fig. 7. Shows a comparison of the capability of dissimilatory arsenate reduction by the *Shewanella* sp. O23S strain in minimal medium R1-R2, having a pH 8 (A) and pH 4 (B). In order to compare the abilities of the investigated strain to dissimilatory reduce arsenates under various pH conditions, anaerobic cultures in minimal R1-R2 medium containing 2.5mM (187.5ppm) sodium arsenate and 5mM sodium lactate were carried out.

DESCRIPTION OF EMBODIMENTS

[0023] The following examples are presented merely to illustrate the invention and to clarify its various aspects, but are not intended to be limitative, and should not be equated with all its scope, which is defined in the appended claims.

[0024] In the following examples, unless it was otherwise indicated, standard materials and methods described in Sambrook et al., 2001. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York, were used, or the manufacturers' instructions for specific materials and methods were followed.

EXAMPLES

Example 1. Characteristics of the plasmid pSheB and determination of its complete sequence

[0025] Plasmid pSheB, of the size of 81 kbp, was isolated from the *Shewanella* sp. O23S strain. In order to sequence the plasmid, plasmid pSheB was isolated from 200 ml of overnight culture of *Shewanella* sp. O23S by alkaline lysis method. Plasmid pSheB was sequenced by pyrosequencing method, using "shotgun" strategy on the GS FLX Titanium (454) sequencer (in the Oligo P1. centre). For the construction of the DNA library, approx. 5 µg of pSheB DNA was used and reagent kits provided by the manufacturer were applied (GS FLX Titanium Library Preparation Kit, Roche). The constructed library was sequenced and assembled using the software from the Newbler de novo assembler package (Roche). The obtained sequences were then assembled into contigs using Seqman software from Lasergene package (DNASTar). Annotation of the plasmid (identification of the open reading frames and determination of their potential functions) were performed using Artemis program and BLAST programs (from the NCBI database). The complete sequence of the plasmid has been shown in the SEQ ID NO: 1. Sequencing of the plasmid pSheB showed that it is a DNA particle of the size 81 591 bp and the GC-content of 44.04%. It comprises 87 open reading frames (ORF), which constitute 89.6% of the sequence of the plasmid. Table 1, below, features a detailed description of the identified ORFs within SEQ ID NO: 1.

Table 1. Determination of the potential coding sequences of the plasmid pSheB in reference to SEQ ID NO: 1. *The numbers in the coding sequence correspond to the nucleotide numbers in SEQ ID NO: 1.

ORF #	Coding sequence (start-stop codon)*	Protein size (aa)	Predicted protein function	Identity (%)	Organism	GenBank number
1	1-813	270	Deoxyribonuclease I (EndA)	97 (233/240)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015569 93
2	826-1061	77	Hypothetical protein	100 (77/77)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	YP_0060186 02
3	1454-3328	624	ParB like nuclease	99 (618/624)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	AEG13584
4	3453-3908	151	Hypothetical protein	99 (149/151)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015569 96
5	4113-4397	94	Hypothetical protein	97(91/94)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	YP_0060185 98
6	4428-4649	73	Hypothetical protein	97(71/73)	<i>Shewanella baltica</i> OS185 (pS18501)	NP_0013554 38
7	5014-5358	114	Hypothetical protein	100 (114/114)	<i>Shewanella baltica</i> OS195 (pS19502)	YP_0015570 25
8	5321-5944	207	Hypothetical protein	99 (204/207)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	YP_0060185 95
9	5946-6347	133	Hypothetical protein	99 (132/133)	<i>Shewanella baltica</i> OS185 (pS18501)	YP_0013554 40
10	6545-6790	81	Toxin protein (HicA)	100 (81/81)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015570 01
11	6790-7125	111	Antitoxin protein (HicB)	100 (111/111)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015570 02
12	7423-7767	114	Hypothetical protein	98 (112/114)	<i>Shewanella baltica</i> OS195 (pS19502)	YP_0015570 25
13	7730-9058	442	Hypothetical protein	98 (4432/442)	<i>Shewanella baltica</i> OS625	EHC04198
14	9656-9967	103	Hypothetical protein	99 (102/103)	<i>Shewanella baltica</i> OS625	EHC04199
15	10260-10445c	61	Hypothetical protein	34 (13/38)	<i>Scheffersomyces stipitidis</i> CBS 6054	XP_0013840 86
16	10524-10883	119	Hypothetical protein	100 (119/119)	<i>Shewanella baltica</i> OS185 (pS18501)	YP_0013554 46
17	10904-16857c	1979	Conjugative transfer relaxase (TraI)	99 (1945/1969)	<i>Shewanella baltica</i> OS195 (pS19502)	YP_0015570 30

(continued)

ORF #	Coding sequence (start-stop codon)*	Protein size (aa)	Predicted protein function	Identity (%)	The greatest similarity (BLASTP program)	GenBank number
18	17109-19235c	708	Type IV conjugative transfer system coupling (TraD)	98 (694/708)	<i>Shewanella baltica</i> OS223 (pS22302)	YP_0023603 31
19	19908-22721c	937	Sex pilus assembly and mating pair (TraG)	95 (892/937)	<i>Shewanella baltica</i> OS223 (pS22302)	YP_0023642 50
20	22724-24112c	462	Type IV conjugative transfer system protein (TraH)	99 (460/462)	<i>Shewanella baltica</i> OS625	EHC04207
21	24296-24736c	146	Type-F conjugative transfer system pilin assembly thiol-disulphide isomerase (TrbB)	97 (141/146)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015569 45
22	24750-25622c	290	Type-F conjugative transfer system pilin assembly protein (TraF)	99 (287/289)	<i>Shewanella baltica</i> OS185 (pS18501)	YP_0013554 54
23	25622-27433c	603	Conjugal transfer mating pair stabilization protein (TraN)	87 (530/607)	<i>Shewanella baltica</i> OS223 (pS22302)	YP_0023642 56
24	27430-28170c	246	Type-F conjugative transfer system pilin assembly protein (TrbC)	100 (246/246)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015569 48
25	28193-29200c	335	Sex pilus assembly and synthesis protein (TraU)	99 (332/335)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	YP_0060170
26	29187-29891c	234	Type-F conjugative transfer system protein (TraW)	99 (232/234)	<i>Shewanella baltica</i> OS625	EHC04215
27	29888-30259c	123	Conjugal transfer protein (TrbI)	98 (120/123)	<i>Shewanella baltica</i> OS223 (pS22302)	YP_0023603 19
28	30261-32849c	862	Type-IV secretion system protein (TraC)	99 (856/862)	<i>Shewanella baltica</i> BA175 (pSBAL17502)	YP_0060265
29	32853-33296c	147	Type IV conjugative transfer system protein (TraV)	100 (147/147)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015569 53
30	33329-34855c	508	Sex pilus assembly and synthesis protein (TraB)	98 (500/508)	<i>Shewanella baltica</i> OS678	YP_0052804 01
31	34852-35667c	271	Type-F conjugative transfer system secretin (TraK)	95 (258/271)	<i>Shewanella baltica</i> OS223 (pS22302)	YP_0023642 64
32	35807-36232c	141	Type IV conjugative transfer system protein (TraE)	97 (118/122)	<i>Shewanella baltica</i> OS223 (pS22302)	YP_0023642 65
33	36273-36575c	100	Type IV conjugative transfer system protein (TraL)	94 (94/100)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015569 57

(continued)

ORF #	Coding sequence (start-stop codon)*	Protein size (aa)	Predicted protein function	Identity (%)	The greatest similarity (BLASTP program)	GenBank number
34	36579-36953c	124	Type IV conjugative transfer system pilin (TraA)	87 (111/128)	<i>Shewanella baltica</i> BA175 (pSBAL17502)	YP_0060228 71
35	37017-37205c	62	Hypothetical protein	100 (62/62)	<i>Shewanella baltica</i> OS223 (pS22301)	YP_0023602 54
36	37307-37564c	85	Hypothetical protein with helix-turn-helix domain	100 (85/85)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015569 59
37	37684-37866	60	Hypothetical protein	100 (60/60)	<i>Shewanella baltica</i> OS195 (pS19501)	YP_0015569 60
38	38139-38321	60	Hypothetical protein	32 (18/56)	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> USA300_FPR3757	YP_494132
39	38308-39240	310	Hypothetical protein	97 (301/310)	<i>Shewanella baltica</i> OS195 (pS19503)	YP_0015571 03
40	39774-40076c	100	Pyridoxamine Kinase family protein	27 (28/104)	<i>Megasphaera micronuciformis</i> F0359	ZP_0775718 7
41	40161-40442c	93	Hypothetical protein	86 (75/87)	<i>Shewanella oneidensis</i> MR-1 (megaplasmid)	NP_720395
42	40448-40975	175	N-Acyltransferase superfamily protein	91 (160/175)	<i>Shewanella oneidensis</i> MR-1 (megaplasmid)	NP_720396
43	41417-41647c	76	Hypothetical protein	100 (74/74)	<i>Shewanella baltica</i> OS223 (pS22301)	YP_0023602 50
44	41822-43012	396	Plasmid partition protein (ParA)	99 (392/396)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	AEG13539
45	43012-44169	385	Plasmid partition protein (ParB)	97 (347/356)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	YP_00601 54
46	44256-44402	48	Hypothetical protein			
47	44438-48298	1268	NTPase with transmembrane helices	25 (321/1284)	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	NP_389778
48	48454-49113	219	Hypothetical protein	60 (131/218)	<i>Methylophaga thiooxydans</i> DMS010	ZP_0510295 2
49	49234-49623	129	Hypothetical protein	39(49/125)	<i>Methylovorus glucosetrophus</i> SIP3-4	YP_0030501 41
50	49640-50545	301	Hypothetical protein	34 (102/300)	delta proteobacterium MLMS-1	ZP_0129054 4

(continued)

ORF #	Coding sequence (start-stop codon)*	Protein size (aa)	Predicted protein function	Identity (%)	The greatest similarity (BLASTP program)	GenBank number
51	50655-51623	322	Hypothetical protein	66 (212/322)	<i>Pseudomonas arctica</i> A 37-1-2	YP_1027990.6
52	51781-52386	201	Hypothetical protein	25 (44/176)	<i>Enterobacter cloacae</i> SCF1	YP_003941396
53	52593-53222c	209	Resolvase domain-containing protein (TnpR)	98 (204/209)	<i>Shewanella baltica</i> OS185 (pS18501)	YP_001355408
54	53345-53827	159	Transposase(TnpA)	72 (32/46)	<i>Vibrio furnissii</i> CIP 102972	EEX38686
55	53836-54123	95	Hypothetical protein	83 (79/95)	<i>Marinomonas</i> sp. MWYL1	ABR70068
56	54532-54840	102	ArsR family transcriptional regulator	94 (96/102)	<i>Shewanella</i> sp. ANA-3	YP_869986
57	54901-55143	80	Thioredoxin - redox-active disulphide protein 2	93 (74/80)	<i>Shewanella</i> sp. ANA-3	YP_869985
58	55162-55695	177	Hypothetical protein	95 (169/177)	<i>Shewanella</i> sp. ANA-3	YP_869984
59	55706-56386	226	Cytochrome c biogenesis protein	99 (225/226)	<i>Shewanella</i> sp. ANA-3	YP_869983
60	56554-57555	333	RND family efflux transporter MFP subunit	93 (311/333)	<i>Shewanella</i> sp. ANA-3	YP_869982
61	57552-60625	1023	Acriflavin resistance protein (AcrB)	98 (1002/1023)	<i>Shewanella</i> sp. ANA-3	YP_869981
62	60743-61177c	144	Arsenate reductase (ArsC)	94 (131/140)	<i>Shewanella</i> sp. ANA-3	YP_869980
63	61265-62512c	415	Arsenical pump membrane protein (ArsB)	98 (407/414)	<i>Shewanella</i> sp. W3-18-1	YP_964320
64	62613-64385c	590	Arsenite-activated ATPase (ArsA)	92 (543/590)	<i>Shewanella</i> sp. W3-18-1	YP_964319
65	64420-64782c	120	Arsenical resistance operon trans-acting repressor (ArsD)	85 (102/120)	<i>Shewanella</i> sp. W3-18-1	YP_964318
66	65155-67719	854	Respiratory arsenate reductase, Mo binding subunit (ArrA)	96 (818/854)	<i>Shewanella</i> sp. ANA-3	YP_869976
67	67731-68435	234	Respiratory arsenate reductase, FeS subunit (ArrB)	97 (228/234)	<i>Shewanella</i> sp. ANA-3	YP_869975
68	68506-69936c	476	Glutathione synthase	86 (409/476)	<i>Shewanella putrefaciens</i> CN-32	YP_001182743
69	70092-71144c	350	Permease	76 (271/358)	<i>Shewanella putrefaciens</i> CN-32	YP_001185328
70	71369-71656	95	ArsR family transcriptional regulator	92 (87/95)	<i>Shewanella</i> sp. ANA-3	YP_869972
71	71755-72171	138	Transcriptional regulator - tyrosine phosphatase (ArsR)	93 (127/138)	<i>Shewanella</i> sp. ANA-3	YP_869971

(continued)

ORF #	Coding sequence (start-stop codon)*	Protein size (aa)	Predicted protein function	Identity (%)	The greatest similarity (BLASTP program)	GenBank number
72	72164-72511	115	ArsR family transcriptional regulator	97 (112/115)	<i>Shewanella</i> sp. ANA-3	YP_869970
73	72590-73102	170	Protein tyrosine phosphatase (ArsC2)	84 (141/167)	<i>Shewanella</i> sp. ANA-3	YP_869969
74	73195-74192	332	Permease	96 (318/332)	<i>Shewanella</i> sp. ANA-3	YP_869968
75	74204-74440	78	Redox-active disulphide protein 2	97 (76/78)	<i>Shewanella putrefaciens</i> CN-32	YP_00118532
76	74448-74939	163	Dual specificity protein phosphatase	86 (140/163)	<i>Shewanella putrefaciens</i> CN-32	YP_00118531
77	74996-76006	336	Glyceraldehyde-3-phosphate dehydrogenase	99 (332/336)	<i>Shewanella</i> sp. W3-18-1	YP_964294
78	76012-77250	412	Major facilitator superfamily protein	99 (409/412)	<i>Shewanella putrefaciens</i> 200	YP_006009170
79	77357-77656	99	Hypothetical protein	92(91/99)	<i>Shewanella putrefaciens</i> CN-32	YP_001185318
80	77964-78146	60	Hypothetical protein	32 (13/41)	<i>Streptococcus pneumoniae</i>	CAI34125
81	78249-78683	144	Peptidase S24/S26A/S26B	97 (139/144)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	YP_006018610
82	78671-79924	417	UMUC domain-containing protein DNA-repair protein	93 (389/417)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	YP_006018609
83	79975-80238	87	Vitamin B 12 dependent methionine synthase	30 (24/81)	<i>Coprococcus comes</i> ATCC 27758	ZP_03798710
84	80344-80994	216	Hypothetical protein	68 (150/219)	<i>Aeromonas hydrophila</i>	YP_0029955
85	81035-81253	72	Hypothetical protein	93 (67/72)	<i>Shewanella baltica</i> OS117 (pSBAL11701)	YP_006035206
86	81273-81591	106	Hypothetical protein	96 (102/106)	<i>Shewanella baltica</i> BA175 (pSBAL17501)	YP_006018603

Example 2. Construction of the plasmid-less strain and functional analysis of the plasmid pSheB

[0026] In order to show, that the plasmid pSheB and the gene module coding for potential proteins located within it, are involved in the resistance to arsenic and dissimilatory arsenates reduction, a plasmid-less derivative of the *Shewanella* sp. O23S strain was constructed and its functional analysis was carried out. As the stress factor stimulating the mechanisms of plasmid removal from the cells of the host, ethidium bromide (EtBr) solution at a final concentration of 5 µM was used.

[0027] Overnight culture of the wild-type *Shewanella* sp. 023 strain, carried out in LB medium supplemented with 5mM sodium arsenate was passaged to LB medium with 5 µM EtBr. The optical density (OD) of the culture at the beginning of the experiment was OD=0.1, and the culture was carried out for 24 hours at 22°C with shaking (160 rpm). After 24 hours of incubation, culture dilutions 10⁻⁴, 10⁻⁶, 10⁻⁸, 10⁻¹⁰ were prepared, respectively, and 100 µl of each of them were plated on LB medium solidified with agar. Subsequently, 96 colonies were randomly selected and passaged by replica plating to:

(i) solid LB medium,

(ii) solid LB medium supplemented with sodium arsenate (50 mM), and

(iii) liquid minimal R1-R2 medium (R1 salt: NaCl-1.17g/l; KCl-0.3g/l; NH₄Cl-0.15g/l; MgCl₂ x 6H₂O-0.41g/l; CaCl₂ x 2H₂O-0.05g/l and R2 salt (KH₂PO₄-0.17g/l; NaHCO₃-2.0g/l; Na₂SO₄ x 10 H₂O-0.07g/l mixed in a ratio 1:1), enriched with sodium lactate at a final concentration of 5mM, salts according to Tuovinen (2ml/l) (pH 6.0; with the composition: Na₂EDTA 50 g/l; ZnSO₄·7H₂O 11 g/l; MnCl₂·7H₂O 5.5 g/l; FeSO₄·7H₂O 2.5 g/l; (NH₄)₆Mo₇O₂₄·4H₂O 5 g/l; CuSO₄·5H₂O 2 g/l; CoCl₂·6H₂O 0.5 g/l; NaOH 11 g/l) (Tuovinen et al., 1973), yeast extract at a final concentration of 0.004% and 2.5mM sodium arsenate.

[0028] The cultures in solid media were incubated for 72 hours, whereas the cultures in the liquid medium were carried out in 200 µl in 96-well titration plates in Anaerocult® (Merck) containers, which provide anaerobic conditions for 168 hours.

[0029] The cultures carried out in the liquid medium were aimed to determine the abilities of the selected strains to dissimilatory reduce arsenates. After 5 days of incubation under anaerobic conditions, 100 µl of 0.1 M solution of silver nitrate were added to the cultures. The result of the reaction between AgNO₃ and As (III) or As (V) is the formation of a coloured precipitate. A brown precipitate indicates the presence of Ag₃AsO₄ (silver orthoarsenate), while a yellow precipitate indicates the presence of Ag₃AsO₃ (silver arsenite). In case of testing for the ability to reduce arsenates, the presence of a yellow precipitate indicates that As(V) was reduced to As(III).

[0030] The cultures carried out in solid LB medium enriched with sodium arsenate were aimed to determine the resistance to As(V). In turn, the cultures carried out in solid LB medium (not enriched with additional substances) were aimed to secure the potential mutants (positive control). All the strains, which have grown in LB medium, and were not capable of growth in LB medium enriched with As(V) and in minimal R1-R2 medium containing arsenate (the final electron acceptor) and lactate (electron donor), have been designated as potential mutants, deprived of the plasmid pSheB.

[0031] In order to verify the selected consortia, the plasmid profile of the wild-type strain (*Shewanella* sp. O23S) and the potential plasmid-less mutants was checked. The plasmid DNA was isolated by alkaline lysis method, and electrophoretic analysis (0.8% agarose gel) was conducted. The comparison of the plasmid patterns of the selected strains allowed for the identification of the plasmid-less mutants. An additional confirmation of the absence of the plasmid pSheB in the cells of the constructed mutants was PCR analysis. PCR reaction was carried out in the genomes of the potential mutants, using the following primers:

end A-L GCTGTTGCTTCCAATACGAC (SEQ ID NO: 2) and

end A-R GGCGCTGCGACTTACTCATC (SEQ ID NO: 3)

Primer end A-L respectively corresponds to the position of the nucleotide 127, while end A-R - position 679 of the plasmid pSheB in reference to SEQ ID NO: 1. The strains (potential mutants) which gave a negative result in the PCR reaction, using the primers described above, were selected for further analysis.

[0032] The next step of the verification of the strains not-possessing the plasmid pSheB was their functional analysis. It was checked again whether the selected strains are capable of dissimilatory arsenate reduction. For this purpose, tests were carried out in minimal R1-R2 medium enriched with sodium arsenate and sodium lactate, and applying the test with 0.1M solution of silver nitrate. The strains, which were not capable of reduction, were susceptible to As(V) and As(III), and did not possess the plasmid pSheB, turned out to be a proof that the plasmid pSheB determines the capability of arsenate respiration and resistance to arsenic. Fig. 2. shows a graph illustrating the kinetics of arsenate reduction of the wild-type strain and the mutant deprived of the plasmid pSheB. The strain deprived of the plasmid was not capable of growth in minimal medium supplemented with 2.5 mM sodium arsenate, thus it was unable to reduce As(V) to As(III). In this way it was shown that the genetic information contained in the plasmid pSheB (SEQ ID NO: 1) determines the acquisition of the capability of growth under anaerobic conditions, using arsenates as the final electron acceptor, thus

the capability of dissimilatory arsenate reduction.

Example 3. Construction of the vector carrying a gene module coding for the proteins involved in dissimilatory arsenate reduction

[0033] In order to demonstrate, which genes located on the plasmid pSheB encode proteins responsible for dissimilatory arsenate reduction, the *arr* module, comprising *i.a.* genes for dissimilatory arsenate reductase *arrAB*, was cloned into the vector pBBR1-MCS2 (Km^r), in the *Escherichia coli* TOP10 strain, and its functionality was tested.

[0034] In order to clone the *arr* module, amplification of a DNA fragment of the size 8634 bp (comprising the region from position 63978 to 72599 in the genome of pSheB) was performed on a DNA template of the plasmid pSheB, isolated by alkaline lysis. For PCR reaction, the following oligonucleotides were used as primers:

She_Mph1103F: GAAATCTTGCACTAGCGATGCAATC (SEQ ID NO: 4) [position in the genome of the plasmid pSheB: 63978 - 64001; the underlined sequence is the restriction site recognized by the enzyme Mph1103I (NsiI)], and
She_XmaJR: *GTTGTTCTTAGG* CTGGTGCCATATCAACCTCTAG (SEQ ID NO: 5) (position in the genome of the plasmid pSheB: 72578 - 72599; the sequence written in *italics* is an added sequence; the underlined site is recognized by the restriction enzyme XmaJI). For the amplification, Phusion® High-Fidelity DNA Polymerase (Thermo Scientific) was used.

[0035] The obtained PCR product (8634bp) was cloned into a plasmid vector: pBBR1MCS-2 (Km^r) [Kovach et al., 1995] digested (linearized) with SmaI. The ligation mixture of the PCR product and the vector pBBR1MCS2 digested with the enzyme SmaI was introduced, by means of chemical transformation, using the calcium-rubidium method according to Kushner (1978), into the cells of *Escherichia coli* Top10 strain [*mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74* *recA1* *araD139* Δ(*ara-leu*)7697 *galU* *galK* *rpsL* *endA1* *nupG*]. Complete LB medium with kanamycin (30 μg/ml), IPTG (0,5 μg), and X-gal (40 μg/ml) was used as the selection medium.

[0036] From the pool of the obtained transformants (white colonies resistant to kanamycin) strains that were harbouring a plasmid of the appropriate size: 13778 bp (pBBR1MCS2 - 5144bp + *arr* module - 8634bp). The presence of the constructed plasmid was confirmed by restriction analysis (digestion with the enzymes XmaJI and Mph1103I), electrophoretic analysis and sequencing. The *Escherichia coli* MR1 strain (derivative of the *E. coli* TOP10 strain) harbouring the plasmid pARR1A (derivative of pBBR1MCS2 with cloned *arr* module), was selected for further analysis.

[0037] In order to verify the functionality of the constructed plasmid pARR1A, phenotypic analysis of the *E. coli* MR1 strain was carried out in minimal R1-R2 medium enriched with 2mM sodium arsenate and 5mM sodium lactate and supplemented with 0.004% yeast extract. The culture was carried out for 120h under anaerobic conditions (in CO₂:N₂ atmosphere) at 37°C. After five days of culturing, the test with 0.1M solution of silver nitrate was carried out, which showed that the investigated strain has reduced arsenates to arsenites. A confirmation of the reduction of arsenates to arsenites by the *E. coli* MR1 (pARR1A) strain, was the qualitative analysis of arsenic speciation by HPLC. The conducted analysis showed that after 120h of incubation of the *E. coli* MR1 strain, arsenites were identified in the medium. In the control reaction with the *E. coli* TOP10 strain (without the plasmid), reduction of As(V) to As(III) was not observed.

[0038] The obtained results showed that the introduction of the genes of the *arr* module, originating from the genome of the plasmid pSheB, on the vector pBBR1MCS into the *E. coli* TOP 10 strain, leads to the acquisition of the capability of dissimilatory arsenate reduction. The module introduced to *E. coli* TOP10 corresponded to the fragment of the nucleotide sequence from 63978 to 72599 of the plasmid pSheB, having the sequence shown in SEQ ID NR: 1. In this way it was demonstrated that this is the fragment of the plasmid responsible for the capability of dissimilatory arsenate reduction. It was also demonstrated, that a bacterium other than that, from which the plasmid pSheB originates, that is another species of bacteria or a bacterial strain, into which the fragment of the sequence comprising the fragment of the nucleotide sequence from 63978 to 72599 of the plasmid pSheB, having the sequence shown in SEQ ID NO: 1 has been introduced, acquires the ability to dissimilatory reduce arsenates.

Example 4. Removal of arsenic from copper minerals using the *Shewanella* sp. O23S strain

[0039] In order to demonstrate that a strain comprising the plasmid pSheB (SEQ ID NO: 1), for example the *Shewanella* sp. O23S strain, harbouring the plasmid pSheB, can be used in biometallurgy, in the processes of selective removal of arsenic from mineral resources, an experiment was carried out, using two types of minerals: copper-bearing bituminous shale of the "Kupferschiefer" type and flotation tailings from the first series, obtained from the Mining Plant "Lubin" (KGHM, Poland). The average arsenic content in the copper-bearing bituminous shale designated as "Shales" was 1000-3000 mg/kg of the shale, whereas the arsenic content in the flotation tailings designated as "Middlings" was 250-350 mg/kg of the tailings. Apart from arsenic, both types of minerals contain Cu in the range of 35000-110000 mg/kg of dry mass, and other precious metals, e.g. Co - 500-2500mg/kg, Zn - 15-2800mg/kg, Ni - 250-500mg/kg.

[0040] The experiment of arsenic removal from the mineral resources described above was conducted in R1-R2 medium enriched with 5mM sodium lactate as the source of carbon and energy, Tuovinen salts (2ml/l) and yeast extract at a final concentration of 0.004%. The mineral substrate (shales or flotation tailings) having the fraction size of 125-250 mm, was added to a final concentration of 10%. The cultures were carried out in 100-ml bottles under anaerobic conditions, in the atmosphere of the mixture of gases N₂:CO₂ (4:1) for 21 days at 22°C with shaking (160rpm). In order to obtain an inoculum of the *Shewanella* sp. O23S strain, overnight cultures were started in liquid complete LB medium. The overnight culture of the O23S strain was centrifuged several times and flushed with saline solution, and then passaged to an appropriate medium, to obtain a density of approximately 10⁶ cells/ml. Sterile medium supplemented with an appropriate substrate, not inoculated with bacteria, was used as the control.

[0041] At the beginning of the experiment, and every 7 days, samples for the determination of copper and arsenic content were collected, which was performed using atomic absorption spectroscopy - flame technique; (AA Solaar M6 Spectrometer, TJA Solutions, UK).

[0042] The conducted analyses showed, that the highest concentration of arsenic in culture fluids in medium enriched with flotation tailings was noted after 14 days of incubation (324.75 µg/l) (Fig.3A). In a further week of incubation, the concentration of arsenic has dropped (192.75 mg/l), which may be associated with precipitation of arsenic in the form of the secondary minerals. In the control samples, concentration of arsenic was at much lower level (it did not exceed 153.75 µg/l), and reflected the chemical processes of leaching. In turn, in culture fluids, in medium enriched with bituminous shales, the highest concentration of arsenic was noted after 21 days of the experiment (678.25 µg/l). At the same time, in the control sample, an approx. 14-times lower concentration of arsenic (47.25 µg/l) was noted. These results indicate that the *Shewanella* sp. O23S strain with the plasmid pSheB (SEQ ID NO: 1) is capable of releasing arsenic from copper minerals. It was therefore extremely important to verify how the release of arsenic would influence copper mobilization. In case of copper content analysis, no significant differences were observed between the Cu content in culture fluids and the control sample. At the beginning of the experiment it was noted that part of the copper was washed out of the minerals as a result of the chemical (stimulated by the medium components) dissolution of copper minerals (Fig. 4). The release of copper occurred with an extremely low efficiency, and Cu concentrations in culture fluids and the control samples did not exceed 3.5 ppm. Furthermore, with the passing of time, the concentration of copper was decreasing (after 21 days of cultivation, the concentration of Cu was below 0.5ppm (Fig. 4.), which was probably associated with the chemical precipitation of copper.

[0043] The conducted experiment allowed to demonstrate that the strain comprising the plasmid pSheB (SEQ ID NO: 1), i.e. the *Shewanella* sp. O23S strain, deposited as KKP2045p, removes arsenic from copper-bearing bituminous shales, as well as from flotation tailings in a specific way, without simultaneous mobilization of copper from copper deposits.

Example 5. Mobilization of arsenic from the soils contaminated with arsenic using the *Shewanella* sp. O23S strain

[0044] In order to demonstrate that a strain harbouring the plasmid pSheB, having the SEQ ID NO: 1 or its functional derivative, such as *Shewanella* sp. O23S, harbouring the plasmid pSheB, can be used in bioremediation, in the processes of selective arsenic removal from the soils contaminated with arsenic, an experiment using the soil originating from the vicinity of a gold mine in Zloty Stok was conducted. The average arsenic content in the soil was 17955.7 mg/kg of soil.

[0045] The experiment of arsenic removal from the soil was conducted in R1-R2 medium enriched with 5mM sodium lactate (carbon and energy source), Tuovinen* salts (2ml/l) and yeast extract at a final concentration of 0.004%. The fragmented sample (having the fraction size of <3mm) was added to a final concentration of 10%. The cultures were carried out in 100-ml bottles under anaerobic conditions in the atmosphere of the mixture of gases N₂:CO₂ (4:1) for 21 days at 22°C with shaking (160rpm). In order to obtain an inoculum of the *Shewanella* sp. O23S strain, overnight cultures were started in liquid complete LB medium. The overnight culture of the O23S strain was centrifuged several times and flushed with saline solution, and then passaged to an appropriate medium to obtain a density of approximately 10⁶ cells/ml. Sterile medium with the addition of the soil, not inoculated with bacteria, was used as the control.

[0046] At the beginning of the experiment, and every 7 days, samples were collected for: (i) determination of the arsenic content (determination by atomic absorption spectroscopy - flame technique) in culture fluids (ii) monitoring the growth of bacteria, by the analysis of the number of colony forming units (cfu) (plating on solid LB medium enriched with 5mM sodium arsenate). The conducted analyses showed, that the highest concentration of arsenic in culture fluids was noted after 14 days of incubation (166.53 mg/l), whereas after a further 7 days of incubation, concentration of arsenic has decreased slightly (123.03 mg/l), which may be associated with the precipitation of arsenic in the form of secondary minerals. In the control samples, concentration of arsenic was at much lower level (it did not exceed 25 mg/l), and reflected the chemical processes of leaching. It was demonstrated that a strain comprising the plasmid pSheB, having the SEQ IN NO:1, i.e. the *Shewanella* sp. O23S strain, removes arsenic from the soils contaminated with this element and can be used in bioremediation, e.g. in the recultivation of arsenic contaminated soils or the removal of arsenic from other contaminated environments.

Example 6. Analysis of arsenic accumulation by the *Shewanella* sp. O23S strain

[0047] The growth experiment and the dissimilatory arsenate reduction performance analysis, carried out in R1-R2 medium (Fig.2) revealed that a strain harbouring the plasmid pSheB, having the SEQ ID NO:1 or its functional derivative, such as *Shewanella* sp. O23S, completely reduces arsenates to arsenites, which are completely removed out of the cell. In order to confirm that the *Shewanella* sp. O23 strain is not capable of accumulating arsenic inside the cells, an additional 24-hour growth experiment was carried out in liquid LB medium. The LB medium was enriched with a solution of sodium arsenites (NaAsO_2) or sodium arsenates (Na_2HAsO_4) having a final concentration of 2 mM. The cultures were carried out in a volume of 50 ml under aerobic conditions at 22°C with shaking (160 rpm). Sterile LB medium with solutions of the investigated salts was used as the control. After 24 hours of incubation, cultures were centrifuged and the arsenic content in culture fluids and biomass (bacterial pellet) was determined by atomic absorption spectroscopy (AA Solaar M6 Spectrometer, TJA Solutions, UK).

[0048] The conducted analyses did not confirm the growth experiments in R1-R2 medium and revealed that arsenic compounds may be partially accumulated inside the cells of the *Shewanella* sp. O23S strain. The efficiency of this process, however is very low, less than 3% for As(III) compounds, and less than 8% for As(V) compounds. The obtain results are shown in Table 2.

Table 2. Comparison of the ability to accumulate arsenic by the *Shewanella* sp. O23S strain, cultured in minimal medium (R1-R2) and complete medium (LB).

	LB medium		R1-R2 medium	
	As(III)	As(V)	As(III)	As(V)
Arsenic content in culture fluids [ppm]	144,8119	163,8377	204,8073	N/D
Arsenic content in the biomass [ppm]	5,809583	13,97905	4,1078	N/D
Accumulation efficiency [%]	3,857076	7,861492	1,9636	N/D
N/D - not determined				

Example 7. Analysis of the production of volatile arsenic compounds by the *Shewanella* sp. O23S strain

[0049] In order to verify whether the *Shewanella* sp. O23S strain comprising the plasmid pSheB is capable of producing volatile arsenic compounds, an anaerobic (in $\text{N}_2:\text{CO}_2$ atmosphere; 4:1) culture in minimal R1-R2 medium, enriched with 5mM sodium lactate and 2.5mM sodium arsenate, was carried out. After 5 days of incubation at 22°C gas samples were collected for the chemical composition analysis in a gas chromatograph GC-MS and GC-AED. The conducted analyses revealed that the only volatile compound produced by the *Shewanella* sp. O23S strain with the plasmid pSheB is dimethyl monosulphide (DMS). The production of volatile arsenic compounds was not observed.

Example 8. Precipitation of As_2S_3 (arsenic(III) sulphide) by the *Shewanella* sp. O23S strain in medium containing As (V) and thiosulfate

[0050] In order to demonstrate that a strain harbouring the plasmid pSheB, having the SEQ ID NO: 1 or its functional derivative, such as *Shewanella* sp. O23S, harbouring the plasmid pSheB can be used in bioremediation, in the processes of selective arsenic removal from the soils contaminated with arsenic, an experiment, in which arsenic was precipitated from the medium in the form of arsenic (III) sulphide (As_2S_3) was carried out. For this purpose, an experiment of arsenic removal from the soil was conducted in R1-R2 medium enriched with 5 mM sodium lactate (as the source of carbon and energy), Tuovinen salts (2ml/l), and 2.5 mM sodium arsenate and 5mM sodium thiosulfate as the final electron acceptors. The medium was inoculated to obtain the initial culture density of 10^6 cells/ml. The cultures were carried out under anaerobic conditions, at room temperature. The arsenic content in the solution was measured at time T0, and on days 1, 2, 3, 4, and 7, 14 and 21 of the culture. The appearance of a yellow-orange arsenic (III) sulphide (As_2S_3) precipitate was also observed. After 7 days, a loss of 47.8% of arsenic in the solution, and the appearance of a characteristic precipitate, insoluble in water, were recorded. After 21 days of cultivation, the loss of arsenic reached 82.3% (Fig.6). In the controls containing a sterile medium, sodium thiosulfate or sulphide, or sulfites (IV) or sulfates (VI), as well as arsenites (III) or arsenates (V), neither the loss of arsenic in the solution, nor the appearance of arsenic sulphide precipitates were observed. It was hereby demonstrated that the strain comprising the plasmid pSheB (SEQ ID NO: 1), i.e. the *Shewanella* sp. O23S strain deposited as KKP2045p, is capable, in the presence of mineral sulfur compounds, of arsenic removal

from solutions (e.g. of soil) in the form of arsenic (III) sulphide, and thus, of its immobilization.

Example 9. Capability of dissimilatory arsenate reduction by the *Shewanella* sp. O23S strain under slightly acidic conditions

[0051] In order to verify whether the *Shewanella* sp. O23S strain is capable of dissimilatory arsenate reduction under slightly acidic conditions, culture was carried out in minimal R1-R2 medium having a pH 4, enriched with 2.5mM sodium arsenate, 5mM sodium lactate and 0.004% yeast extract. The culture was carried out for 168h at room temperature. An analogous experiment in medium having a pH 8 was carried out as the control. The conducted research revealed, that in the most optimal conditions, in medium having a pH 8, *Shewanella* sp. O23S has completely reduced As(V) present in the medium within 4h (Fig. 7A). On the other hand, in medium having a pH 4, *Shewanella* sp. O23S metabolism, and the kinetics of the process of reduction of arsenates to arsenites have been slowed down, and the complete reduction of As(V) occurred after 48h (Fig. 7B). Nevertheless, the obtained results revealed that the *Shewanella* sp. O23S strain is capable of dissimilatory arsenate reduction under slightly acidic conditions.

Example 10. Resistance to heavy metals of the *Shewanella* sp. O23S strain

[0052] In order to use a strain in mobilization of arsenic from polymetallic deposits, copper concentrates or flotation tailings, the used strain must be characterized by a high resistance to heavy metals. In accordance with the above, *Shewanella* sp. O23S capability of tolerating the presence of heavy metals, and capability of reducing As(V) to As(III) in the presence of heavy metals was verified.

[0053] In order to verify the range of tolerance to the presence of heavy metals, the *Shewanella* sp. O23S strain was cultured in liquid complete LB medium supplemented with the appropriate heavy metals solutions (Table 3):

Table 3. Metals and their compounds used for the determination of the minimal inhibitory concentration (MIC).

Analysed metal	Chemical compound	Concentration [mM]	Concentration [mg/l]
As(III)	NaAsO ₂	0,5-5	37,5 - 375
As(V)	Na ₂ HAsO ₄	1-600	75 - 45 000
Cr (III)	Cr ₂ (SO ₄) ₃ ·18H ₂ O	2-12	104 - 624
Zn (II)	ZnSO ₄ ·7H ₂ O	1-6	65,5 - 393
Se (VI)	Na ₂ SeO ₄ ·10H ₂ O	1-20	79 - 1580
Cu (II)	CuSO ₄ ·5H ₂ O	1-6	63,5 - 380
Co (II)	CoSO ₄ ·7H ₂ O	1-6	59 - 354
Mn (II)	MnSO ₄ ·H ₂ O	1-20	55 - 330
V (V)	NaVO ₃	1-20	51 - 1020
Cd (II)	CdSO ₄ ·8H ₂ O	1-6	112 - 672

[0054] The overnight culture of *Shewanella* sp. O23S was passaged to LB medium enriched with the appropriate metal or metalloid compound (to obtain a density of approximately 10⁶ cfu/ml) and was incubated for 24 hours at 22°C. Subsequently, OD₆₀₀ measurements were carried out and the values of minimal inhibitory concentration (MIC), which is defined as the lowest Meⁿ⁺ concentration that completely inhibits the growth of bacteria, were determined. In order to verify whether the presence of heavy metals inhibits the capability of reducing arsenates to arsenites, a test was carried out in minimal R1-R2 medium enriched with 2.5mM sodium arsenate, 5mM sodium lactate, 0.004% yeast extract and the addition of the appropriate heavy metals solutions.

[0055] The conducted experiments revealed that the *Shewanella* sp. O23S strain, apart from the resistance to As(III) and As(V) is also resistant to Cu, Cd, Cr, Co, Mn, Zn, Se, V (Table 4). Furthermore, it is also capable of dissimilatory arsenate reduction in the presence of heavy metals. Only in the presence of iron (III) the capability of dissimilatory reduction was not demonstrated, because this strain is capable of Fe(III) reduction and using it as the final electron acceptor (Table 4).

Table 4. The value of the minimal concentration of heavy metals, inhibiting the growth of bacteria (MIC) and the value of the maximum concentration of metals allowing for dissimilatory reduction of 2.5mM sodium arsenate by the *Shewanella* sp. O23S strain.

	Element	MIC value [mM]	Reduction of arsenic in the presence of the metal [mM]
5	Cd(II)	1	1
	Co(II)	2	2
	Cr(III)	5	3
10	Cu(II)	3	2
	Fe(III)	5	-
	Mn(II)	>20	>5
	Se(VI)	>20	5
15	Zn(II)	3	2
	V(V)	>20	>5

[0056] Literature in the description, as cited herein:

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In the following sequence listing:

[0057]

- SEQ ID NO: 1 corresponds to the complete nucleotide sequence of the plasmid pSheB of *Shewanella* sp. O23S

SEQUENCE LISTING

[0058]

<110> University of Warsaw

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Claims

1. An isolated plasmid pSheB, having the sequences shown in SEQ ID NO: 1.
2. An isolated plasmid comprising the nucleotide sequence from position 63978 to 72599 of the plasmid pSheB according to claim 1.
3. A bacterial strain comprising the plasmid defined in claim 1 or 2, or comprising a nucleotide sequence comprising the nucleotides from 63978 to 72599 of SEQ ID NO: 1.
4. The bacterial strain according to claim 3, **characterised in that** it is the *Shewanella* sp. O23S strain deposited in the IAFB Collection of Industrial Microorganisms in Warsaw, under the deposit number KKP 2045p.
5. A composition comprising the isolated plasmid defined in claims 1-2 or the bacterial strain defined in claims 3-4 or combinations thereof.
6. Use of the bacterial strain defined in claims 3-4 and/or the composition defined in claim 5 for the selective removal of arsenic from mineral resources, raw materials, industry waste or soil, wherein preferably the mineral resources are polymetallic copper deposits.
7. A method for selective removal of arsenic from mineral resources, raw materials, industry waste or soil, wherein the step of dissimilatory arsenate reduction is carried out using the bacterial strain defined in claims 3-4 and/or the composition defined in claim 5.

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8. The method, according to claim 7, wherein the step of dissimilatory arsenate reduction is carried out under neutral or slightly alkaline conditions.

9. The method according to claims 7-8, **characterised in that** the mineral resources are copper deposits.

10. The method according to claim 8, wherein the removal of arsenic is carried out by dissimilatory arsenate reduction using a bacterial strain comprising the plasmid pSheB having the sequence shown in SEQ ID NO: 1, preferably the *Shewanella* sp. O23S strain deposited as KKP2045p, said method comprising the following steps:

- (a) preparing of the mineral resources, wastes or soils and mixing with an appropriate culture medium to enable the cultivation of the strain,
- (b) adding an inoculum of said strain and carrying out the cultivation under conditions enabling the growth of said strain and the conduction of dissimilatory arsenate reduction.

11. The method according to claim 10, **characterized in that**, step (b) is followed by step

- (c) selectively removing arsenic, released from the solutions obtained in step (b), wherein preferably the removal of the released arsenic is carried out by flotation, more preferably by selective precipitation of arsenites with sulphides.

12. The method according to claims 10-11, **characterized in that**:

- step (a) is carried out by shredding and fractionation of the mineral resources, wastes and soils, preferably to the fraction of 125-250 μ m.

13. The method according to claims 10-12, **characterized in that** the culture medium is R1-R2 medium, supplemented with sodium lactate, yeast extract and Tuovinen salts.

14. The method according to claims 10-13, **characterized in that**, the culture medium does not contain NO₃⁻ and Fe³⁺.

15. The method according to claims 10-14, **characterized in that** in step (b), the culture is carried out under anaerobic atmosphere conditions, and is carried out with flushing of the medium with a mixture of gases N₂:CO₂, preferably in a ratio 4:1.

Patentansprüche

1. Isoliertes Plasmid pSheB mit der in SEQ ID NO: 1 gezeigten Sequenz.

2. Isoliertes Plasmid umfassend das Fragment der Nukleotidsequenz von 63978 bis 72599 des Plasmids pSheB nach Anspruch 1.

3. Bakterienstamm umfassend das in Anspruch 1 oder 2 definierte Plasmid, oder umfassend eine Nukleotidsequenz umfassend die Nukleotide von 63978 bis 72599 von SEQ ID NO: 1.

4. Bakterienstamm nach Anspruch 3, **dadurch gekennzeichnet, dass** er der *Shewanella* sp. O23S Stamm ist, hinterlegt in der IAFB Sammlung von Industriellen Mikroorganismen in Warschau unter der Depotnummer KKP 2045p.

5. Zusammensetzung umfassend das in Ansprüchen 1 - 2 definierte isolierte Plasmid oder den in Ansprüchen 3 - 4 definierten Bakterienstamm oder eine Kombination davon.

6. Verwendung des in den Ansprüchen 3 - 4 definierten Bakterienstammes und/oder der in Anspruch 5 definierten Zusammensetzung zur selektiven Entfernung von Arsen aus mineralischen Ressourcen, Rohstoffen, Industrieabfällen oder Boden, wobei die mineralischen Ressourcen vorzugsweise polymetallische Kupfervorkommen sind.

7. Verfahren zur selektiven Entfernung von Arsen aus mineralischen Ressourcen, Rohstoffen, Industrieabfällen oder Boden, wobei der Schritt der dissimilatorischen Arsenreduktion mit dem in Ansprüchen 3 - 4 definierten Bakterienstamm und/oder der in Anspruch 5 definierten Zusammensetzung durchgeführt wird.

8. Verfahren nach Anspruch 7, wobei der Schritt der dissimilatorischen Arsenreduktion unter neutralen oder leicht alkalischen Bedingungen durchgeführt wird.

9. Verfahren nach Ansprüchen 7 - 8, **dadurch gekennzeichnet, dass** die mineralischen Ressourcen Kupfervorkommen sind.

10. Verfahren nach Anspruch 8, wobei die Entfernung von Arsen durch dissimilatorische Arsenreduktion durchgeführt wird, unter Verwendung eines Bakterienstammes umfassend das Plasmid pSheB mit der Sequenz gezeigt in SEQ ID NO: 1, vorzugsweise des *Shewanella* sp. O23S Stammes hinterlegt als KKP 2045p, dieses Verfahren umfassend die folgenden Schritte:

(a) Aufbereiten der mineralischen Ressourcen, Abfälle oder Böden und Mischen mit einem geeigneten Nährmedium um die Kultivierung des Stammes zu ermöglichen,

(b) Hinzufügen einer Impfkultur von diesem Stamm und Durchführen der Kultivierung unter Bedingungen die das Wachstum des Stammes und die Durchführung der dissimilatorischen Arsenreduktion ermöglichen.

11. Verfahren nach Anspruch 10, **dadurch gekennzeichnet, dass** Schritt (b) gefolgt wird von Schritt (c) selektives Entfernen von Arsen, freigesetzt aus den Lösungen erhalten in Schritt (b), wobei vorzugsweise die Entfernung des freigesetzten Arsens durch Aufschwämmen durchgeführt wird, weiter bevorzugt durch selektive Fällung des Arsens mit Sulfiden.

12. Verfahren nach Ansprüchen 10 - 11, **dadurch gekennzeichnet, dass**:

Schritt (a) durch Zerkleinerung und Fraktionierung der mineralischen Ressourcen, Abfälle und Böden durchgeführt wird, bevorzugt in eine Fraktion von 125 bis 150 μm .

13. Verfahren nach Ansprüchen 10 - 12, **dadurch gekennzeichnet, dass** das Nährmedium R1-R2 Medium ist, ergänzt mit Natriumlaktat, Hefeextrakt und Tuovinsalze.

14. Verfahren nach Ansprüchen 10 - 13, **dadurch gekennzeichnet, dass** das Nährmedium kein NO_3^- und Fe^{3+} enthält.

15. Verfahren nach Ansprüchen 10 - 14, **dadurch gekennzeichnet, dass** in Schritt (b) die Kultur unter anaeroben atmosphärischen Bedingungen durchgeführt wird, und durchgeführt wird mit Durchspülung des Mediums mit einer Mischung von Gasen $\text{N}_2:\text{CO}_2$, bevorzugt in einem Verhältnis von 4:1.

Revendications

1. Plasmide isolé pSheB, ayant la séquence représentée par SEQ ID NO : 1.

2. Plasmide isolé, comprenant la séquence nucléotidique à partir de la position 63 978 à 72 599 du plasmide pSheB selon la revendication 1.

3. Souche bactérienne, comprenant le plasmide défini dans la revendication 1 ou 2, ou comprenant une séquence nucléotidique comprenant les nucléotides allant de 63 978 à 72 599 de SEQ ID NO : 1.

4. Souche bactérienne selon la revendication 3, **caractérisée en ce que** c'est la souche *Shewanella* sp. O23S déposée dans la Collection IAFB de micro-organismes industriels de Varsovie, sous le numéro de dépôt KKP 2045p.

5. Composition comprenant le plasmide isolé défini dans les revendications 1 et 2 ou la souche bactérienne définie dans les revendications 3 et 4 ou leurs combinaisons.

6. Utilisation de la souche bactérienne définie dans les revendications 3-4 et/ou de la composition définie dans la revendication 5 pour éliminer sélectivement l'arsenic de ressources minérales, de matières premières, de déchets industriels ou de sols, dans laquelle, de préférence, les ressources minérales sont des dépôts de cuivre polymétallique.

7. Procédé d'élimination sélective de l'arsenic de ressources minérales, de matières premières, de déchets industriels

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ou de sols, dans lequel l'étape de réduction dissimilatrice de l'arséniate est réalisée en utilisant la souche bactérienne définie dans les revendications 3 et 4 et/ou la composition définie dans la revendication 5.

5 8. Procédé selon la revendication 7, dans lequel l'étape de réduction dissimilatrice de l'arséniate est réalisée en condition neutre ou légèrement alcaline.

9. Procédé selon les revendications 7-8, **caractérisé en ce que** les ressources minérales sont des dépôts de cuivre.

10 10. Procédé selon la revendication 8, dans lequel l'élimination de l'arsenic est réalisée par la réduction dissimilatrice de l'arséniate en utilisant une souche bactérienne comprenant le plasmide pSheB ayant la séquence représentée par SEQ ID NO : 1, de préférence la souche *Shewanella* sp. O23S déposée sous le numéro KKP 2045p, ledit procédé comprenant les étapes suivantes :

15 (a) la préparation des ressources minérales, des déchets ou des sols et le mélange avec un milieu de culture approprié pour permettre la culture de la souche,

(b) l'ajout d'un inoculum de ladite souche et la mise en oeuvre de la culture dans des conditions permettant la croissance de ladite souche et la mise en oeuvre d'une réduction dissimilatrice de l'arséniate.

20 11. Procédé selon la revendication 10, **caractérisé en ce que** l'étape (b) est suivie par l'étape (c) d'élimination sélective de l'arsenic, libéré des solutions obtenues dans l'étape (b), dans laquelle de préférence, l'élimination de l'arsenic libéré est réalisée par flottation, de manière plus préférée par précipitation sélective des arsénites avec des sulfures.

25 12. Procédé selon les revendications 10-11, **caractérisé en ce que** :

l'étape (a) est réalisée par déchetage et fractionnement des ressources minérales, des déchets et des sols, de préférence jusqu'à la fraction de 125 μm à 250 μm .

30 13. Procédé selon les revendications 10-12, **caractérisé en ce que** le milieu de culture est le milieu R1-R2 complété avec du lactate de sodium, un extrait de levure et des sels de Tuovinen.

14. Procédé selon les revendications 10-13, **caractérisé en ce que** le milieu de culture ne contient pas de NO_3^- et de Fe^{3+} .

35 15. Procédé selon les revendications 10-14, **caractérisé en ce que**, dans l'étape (b), la culture est réalisée dans des conditions d'atmosphère anaérobie, et est réalisée en rinçant le milieu avec un mélange de gaz N_2 : CO_2 , de préférence à un rapport de 4 : 1.

Fig. 1.

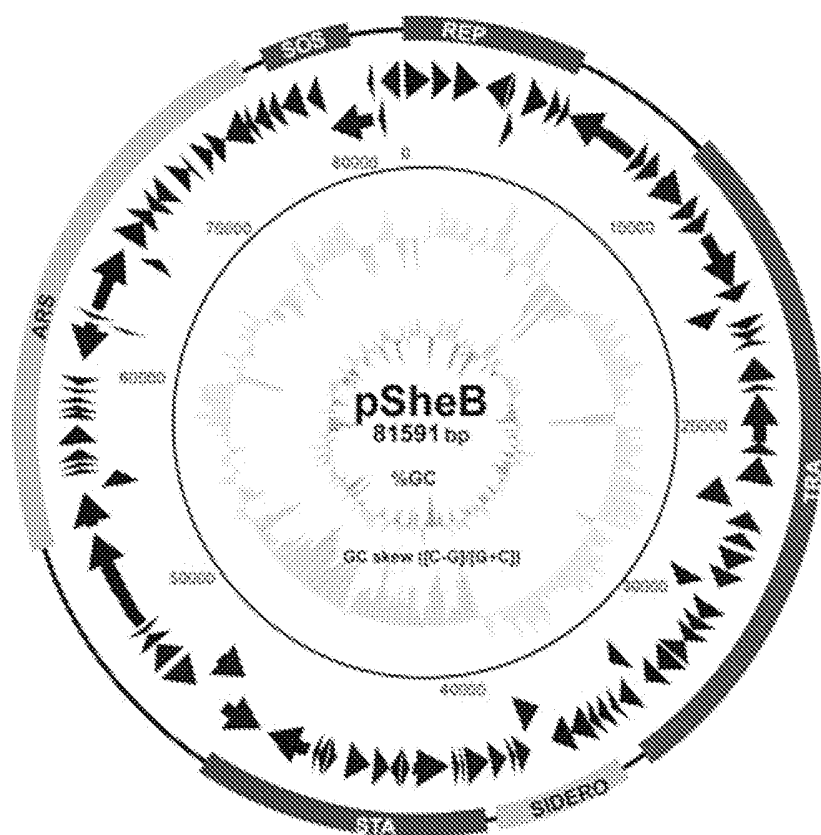


Fig. 2.

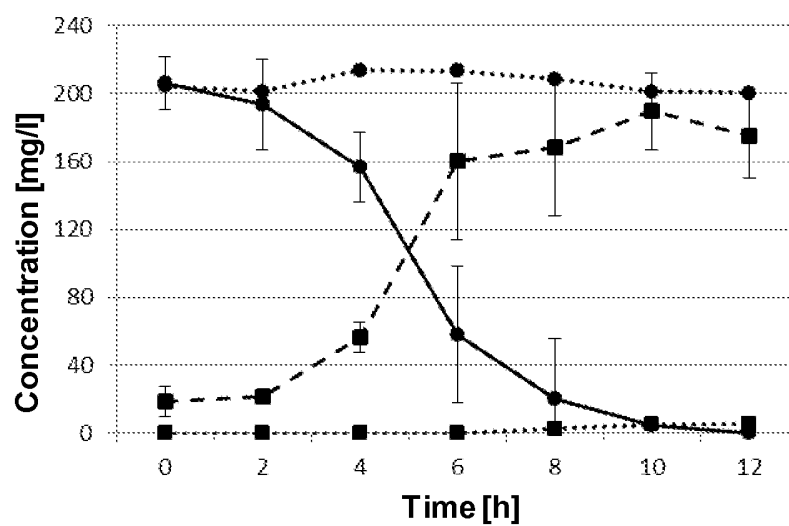


Fig. 3.

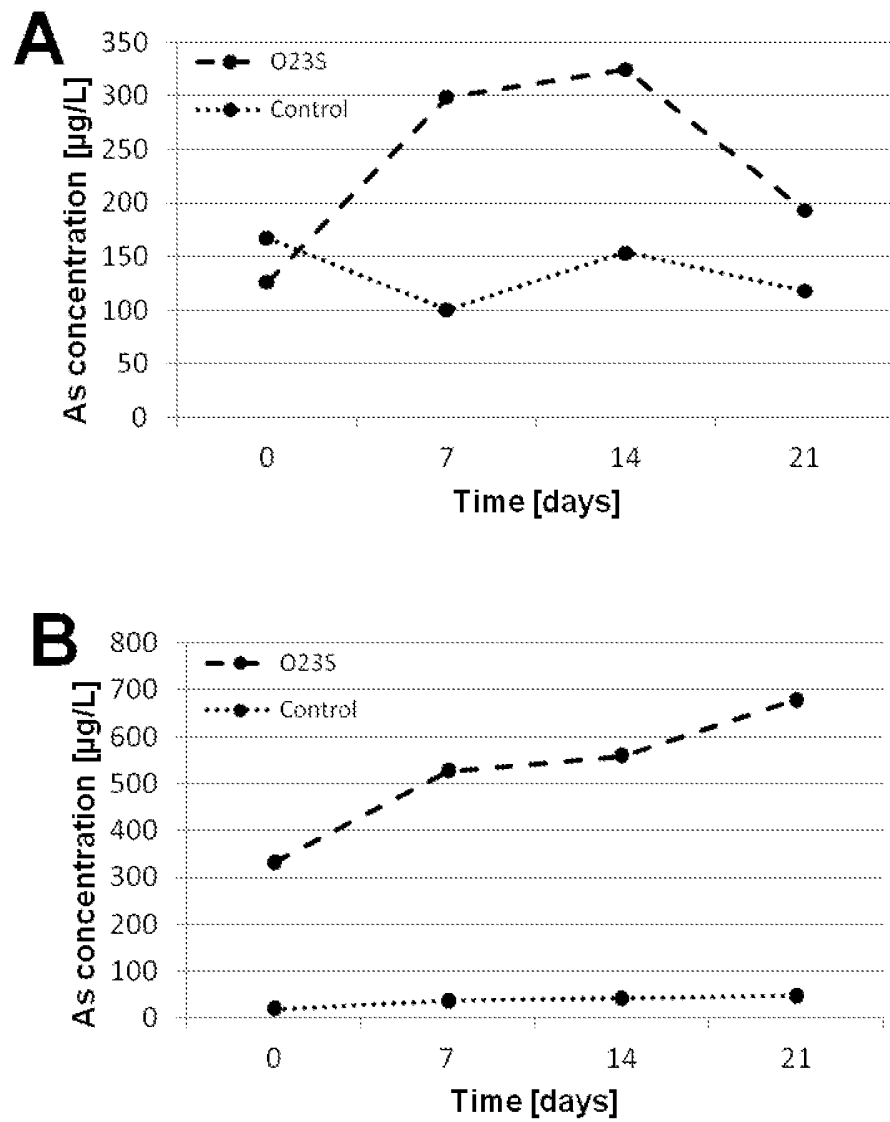


Fig. 4.

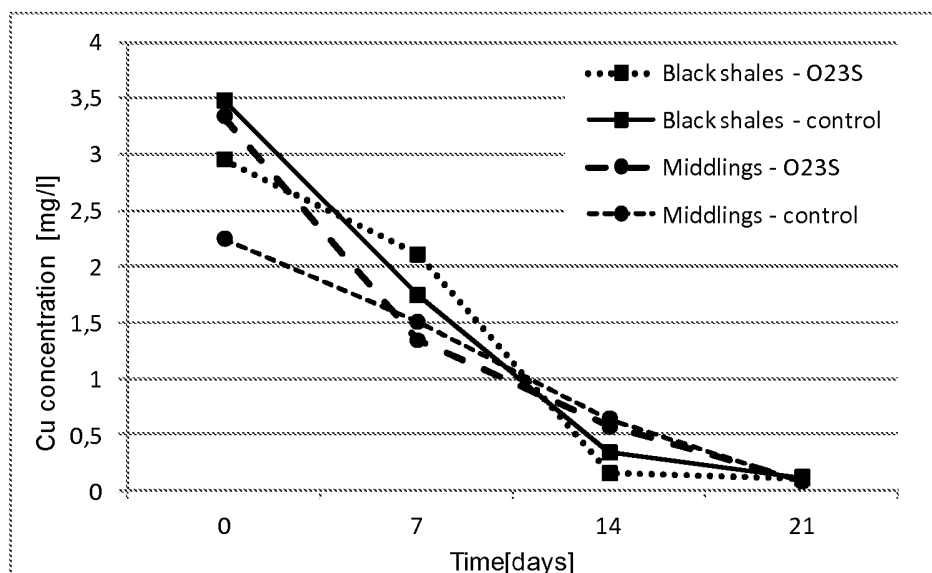


Fig. 5.

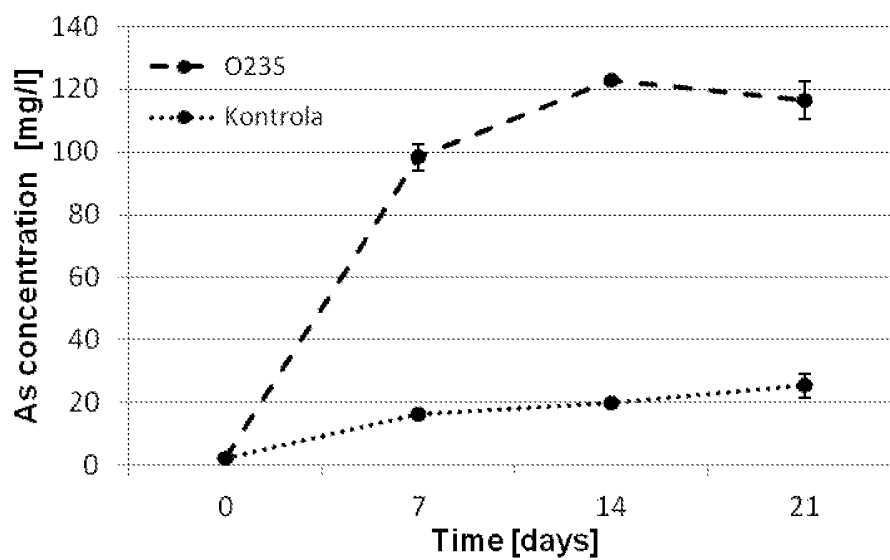


Fig.6

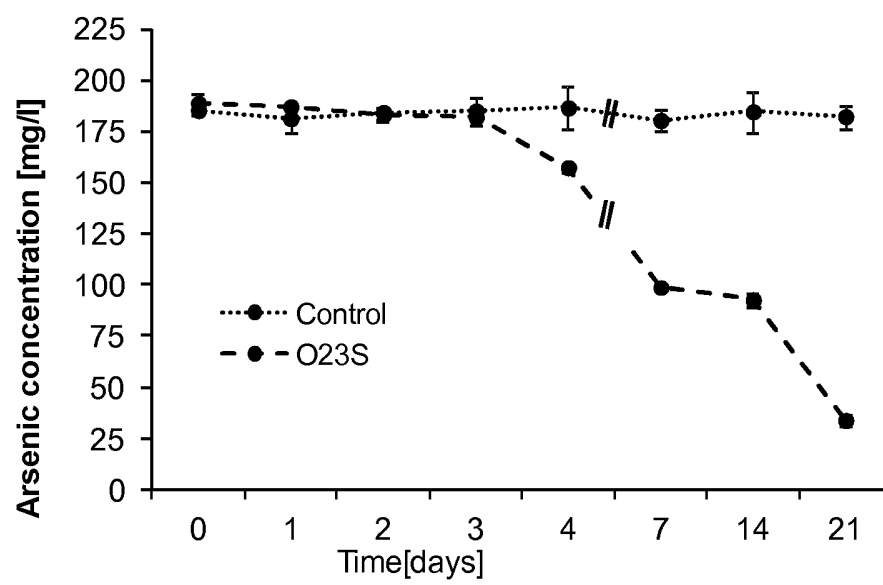
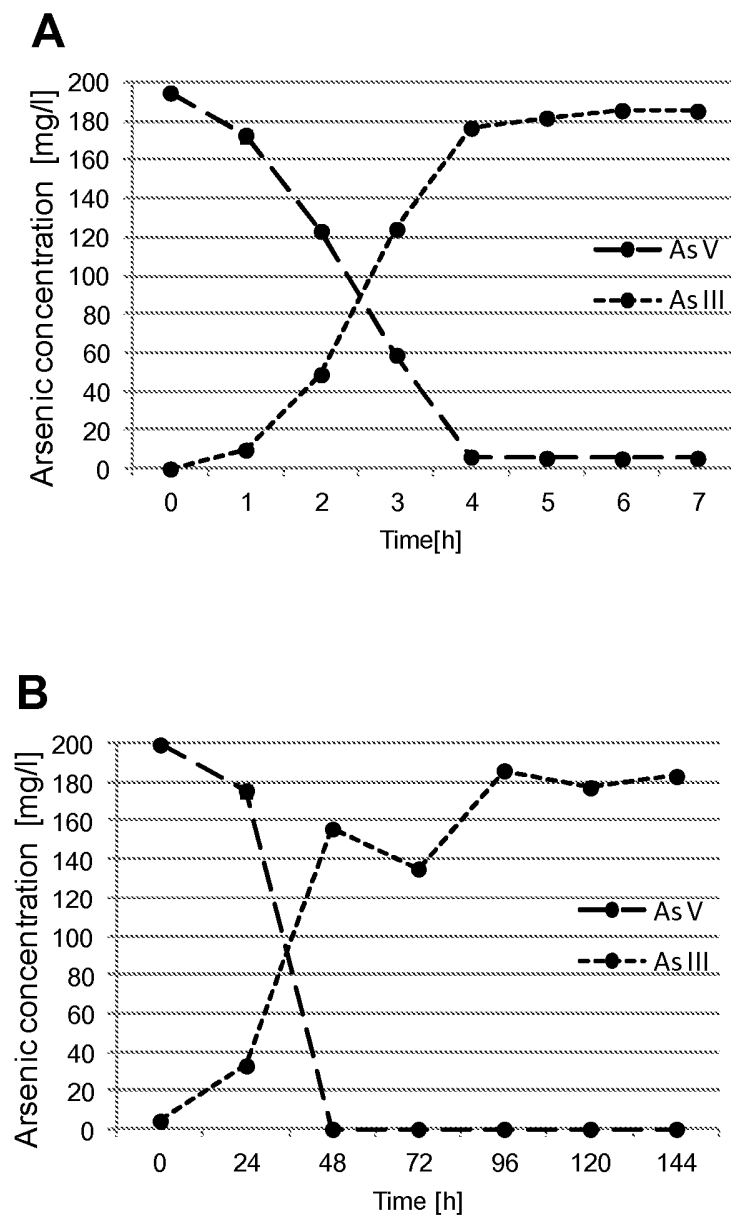


Fig. 7



REFERENCES CITED IN THE DESCRIPTION

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**ARZÉN ELTÁVOLÍTÁSA DISSZIMILÁCIÓS ARZÉN REDUKTÁZ ALKALMAZÁSÁVAL
SZABADALMI IGÉNYPONTOK**

1. pSheB izolált plazmid, amelynek szekvenciája a SEQ ID NO:1-ben van bemutatva.
2. Izolált plazmid, amely tartalmazza azt a nukleotid szekvenciát, amely az 1. igénypont szerinti pSheB plazmid 63978 és 72599 pozíciói között található.
3. Baktériumtörzs, amely tartalmazza az 1. vagy 2. igénypontban meghatározott plazmidot, vagy amely tartalmaz egy olyan nukleotid szekvenciát, amely magában foglalja a SEQ ID NO:1 azon nukleotidjait, amelyek a SEQ ID NO:1 63978 és 72599 pozíciói között találhatók.
4. A 3. igénypont szerinti baktériumtörzs, **azzal jellemezve, hogy ez az O23S jelű Shewanella sp. törzs**, amely letétbe van helyezve (deponálva van) a varsói ipari mikroorganizmus törzsgyűjteményben (IAFB Collection of Industrial Microorganisms in Warsaw) KKP 2045p letéti számon (deposit number).
5. Kompozíció, amely tartalmazza az 1. vagy 2. igénypontban meghatározott izolált plazmidot vagy a 3. vagy 4. igénypontban meghatározott baktériumtörzset, vagy ezek kombinációt.
6. Alkalmazása a 3. vagy 4. igénypontban meghatározott baktériumtörzsnek és/vagy az 5. igénypontban meghatározott kompozíciónak arzén (arsenic) szelektív eltávolítására ásványi forrásokból (resource), nyersanyagokból, ipari hulladékokból, vagy talajból, ahol az ásványi források előnyösen többfémes (polymetallic) réz lerakatok (deposit).
7. Eljárás arzén szelektív eltávolítására ásványi forrásokból, nyersanyagokból, ipari hulladékokból, vagy talajból, ahol a disszimilációs (dissimilatory) arzenát redukció lépését azon baktériumtörzs alkalmazásával hajtjuk végre, amely a 3. vagy 4. igénypontban van meghatározva, és/vagy olyan kompozíció alkalmazásával, amely az 5. igénypontban van meghatározva.
8. A 7. igénypont szerinti eljárás, ahol a disszimilációs arzenát redukciót semleges vagy enyhén alkalikus körülmények között hajtjuk végre.
9. A 7. vagy 8. igénypont szerinti eljárás, **azzal jellemezve, hogy az ásványi források réz lerakatok.**
10. A 8. igénypont szerinti eljárás, ahol az arzén eltávolítását disszimilációs arzenát redukció alkalmazásával hajtjuk végre olyan baktériumtörzset alkalmazva, amely tartalmazza a pSheB plazmidot, amely pedig rendelkezik a SEQ ID NO:1-ben bemutatott szekvenciával; ez a törzs előnyösen a az O23S jelű Shewanella sp. törzs, amely letétbe van helyezve KKP 2045p letéti számon (deposit number); ez az említett eljárás a következő lépéseket tartalmazza: előkészítjük az ásványi forrásokat, hulladékokat vagy talajt, és összekeverjük megfelelő tenyésztő táphözeggel (culture medium), hogy lehetővé váljék a törzs tenyésztése; hozzáadjuk az említett törzs inkulmát és végrehajtjuk a tenyésztést olyan körülmények között, amely lehetővé teszi az említett törzs növekedését és a disszimilációs arzenát redukció végrehajtását.
11. A 10. igénypont szerinti eljárás, **azzal jellemezve, hogy a (b) lépést további lépés követi:**

(c) szelektíven távolítjuk el azt az arzént, amely a (b) lépésben nyert oldatból bocsátódott ki, ahol a kibocsátott arzén eltávolítását előnyösen flotálással, még előnyösebben az arzenitek szulfidokkal történő szelektív kicsapásával hajjuk végre.

12. A 10. vagy 11. igénypont szerinti eljárás, **azzal jellemezve, hogy**

az (a) lépést az ásványi források, a hulladékok és a talaj feldarabolása (shredding) és frakcionálása után hajjuk végre, előnyösen a 125 µm és 250 µm közötti mérettartományban levő frakcióba.

13. A 10-12. igénypontok bármelyike szerinti eljárás, **azzal jellemezve, hogy a tenyésztő tápközeg R1-R2 tápközeg, amely ki van egészítve nátrium-laktáttal, élesztőkivonattal és Tuovinen-sókkal.**

14. A 10-13. igénypontok bármelyike szerinti eljárás, **azzal jellemezve, hogy a tenyésztő tápközeg nem tartalmaz NO_3^- és Fe^{3+} ionokat.**

15. A 10-14. igénypontok bármelyike szerinti eljárás, **azzal jellemezve, hogy a (b) lépésben a tenyésztést az anaerob atmoszféra körülményei között hajjuk végre, és ezt a tápközeg átbuborékolásával (flushing) hajjuk végre N_2/CO_2 gázok keverékével, amelyek előnyösen 4:1 arányban vannak jelen.**