

## USING SYNTHETIC LIXIVANT BIOLOGY FOR THE RECOVERY OF PRECIOUS AND TOXIC METALS FROM ANTHROPOGENIC SOURCES

### FIELD OF THE INVENTION

The present invention relates to a method of using enzymes to break down  
5 cyanide, and a method of synthetic metal recovery for synthetic lixiviant biology. More particularly, the invention relates to the use of engineered bacteria to be used in processes involving cyanogenesis, reduction of leached metal ions, cyanolysis and downstream recycling of cyanide. Further provided are tools for transcription control in *C. violaceum*.

### 10 BACKGROUND OF THE INVENTION

Cyanide readily combines with most major and trace metals to form cyanide complexes, a property which makes it useful in extracting metals from ores. Sodium cyanide is most commonly used in mining sites which readily dissolves in water, yielding sodium ion and cyanide ion,  $\text{CN}^-$ . Some  $\text{CN}^-$  will convert into hydrogen cyanide, HCN  
15 and their relative amounts are determined by the water pH. At pH above 9.0,  $\text{CN}^-$  is the predominant stable form. As pH drops, there will be increasing amount of  $\text{CN}^-$  converting to HCN which readily forms a gas and release into the air. Hence, most mining solutions are maintained at pH above 10.0 which prevents formation of HCN gas and accidental poisoning of mine workers via inhalation. As cyanide is carbon based, it reacts readily  
20 with other carbon-based matter, making it toxic to many living organisms. Hence, cyanide-containing waste must be detoxified before disposal. Conventional methods include alkaline chlorination which is both hazardous and costly. Furthermore, problems arise when the cyanide used in mining does not break down quickly into harmless substances. Even these less toxic substances can persist in the environment for a  
25 significant period of time which can pose problems to the aquatic ecosystem.

The electronic waste recycling industry uses chemical processes which pose considerable environmental risks: current processes for recovering precious metals such as gold, and removal of toxic metals such as lead and mercury, include pyrometallurgy (open burning, etc.) and hydrometallurgy (acid leaching and industrial cyanidation or

cyanide baths); these methods are energy intensive, require additional electrolysis steps for metal separation, and are extremely pollutive in nature (Korte, F., Spiteller, M. & Coulston, F. (2000) *Ecotoxicology and Environmental Safety* 46, 241-245; Fields, S. (2001) *Environ Health Perspect* 109, A474-481). There have been research efforts in

5 using biotechnological leaching processes to replace the industrial chemical processes so that the metal recovery and remediation is simpler, more cost efficient and environmentally benign, and scientists and engineers such as Brandl (Brandl, H., Lehmann, S., Faramarzi, M. A., and Martinelli, D. (2008), *Hydrometallurgy* 94, 14-17), Watling (Watling, H. R. (2006), *Hydrometallurgy* 84, 81-108) and Rawlings (Rawlings, D.

10 E. (2002), *Annual Review of Microbiology* 56, 65-91) have contributed significantly to the field of biotechnological leaching. Compared to conventional techniques for precious metal recovery via acid solubilisation, current efforts in bioremediating electronic waste to recover precious metals such as gold involve the use of lixiviant-producing microbes (Korte, F., Spiteller, M. & Coulston, F. (2000) *Ecotoxicology and Environmental Safety*

15 46, 241-245; Pham, V., and Ting, Y. P. (2009), *Advanced Materials Research* 71, 661-664; Liang, G., Mo, Y., and Zhou, Q. (2010), *Enzyme and Microbial Technology* 47, 322-326; Chi, T. D., Lee, J. C., Pandey, B. D., Yoo, K., and Jeong, J. (2011), *Miner Eng* 24, 1219-1222). In such microbes, the lixiviant involved in bioremediation and recovery of metals is usually hydrogen cyanide. Although possible hydrogen cyanide leakage poses

20 a considerable threat to the environment, the use of microbes in the biomining industry limits and minimizes such concerns as the microbes in context are both cyanogenic (capable of generating cyanide equivalents) and cyanolytic (capable of detoxifying cyanide equivalents), virtually ensuring that there will be no bulk release of cyanide into the environment during the bioleaching process.

25 In contrast to existing processes, bioleaching by naturally-occurring microorganisms, working under mild operating conditions, may allow metal recycling in a process analogous to natural biogeochemical cycles, and hence reduces the demand for resources such as ores, energy or landfill space (Brandl, H., Lehmann, S., Faramarzi, M. A., and Martinelli, D. (2008), *Hydrometallurgy* 94, 14-17). Bioleaching is of interest

30 since it represents "clean technology". As a leaching agent, hydrogen cyanide is formed by a variety of bacteria (e.g. *Chromobacterium violaceum*, *Pseudomonas fluorescens*,

and *P. aeruginosa*) and fungi (e.g. *Marasmius oreades*, *Clitocybe* sp., *Polysporus* sp.) (Pham, V., and Ting, Y. P. (2009), *Advanced Materials Research* 71, 661-664). Cyanide is formed as a secondary metabolite and for a short duration within the lifetime of the microbe. Although cyanide production by microorganisms is known for many years, quantitative data on cyanide production for many species are lacking (Liang, G., Mo, Y., and Zhou, Q. (2010), *Enzyme and Microbial Technology* 47, 322-326). However, current biorecovery and bioremediation efforts are unable to match industrial expectations of cost efficiency (Faramarzi, M. A., et al., (2004), *Journal of Biotechnology* 113, 321-326; Krebs, W., et al., (1997), *FEMS Microbiology Reviews* 20, 605-617) (efficient metal recovery, short time of bioleaching, and non-reliance on conventional electrolysis for metal separation) of leaching, resulting in the continued use of conventional hydrometallurgical and pyrometallurgical methods for metal remediation. This disparity in expectations is a result of the inherent sub-optimal lixiviant metabolism of microbes, and the absence of a suitable biological reduction route for specific metal recovery after bioleaching.

There is a pressing need to develop sustainable technologies to recycle electronic waste in order to protect our environment and preserve natural resources. The invention focuses on the recovery of precious metals and the removal of toxic metals from electronic waste. Current conventional treatment technologies for electronic waste using strong acids or cyanide are pollutive in nature.

## SUMMARY OF THE INVENTION

The present invention generally relates to methods of biological reduction of metal-cyanide complexes after metal-cyanidation and methods of biologically hydrolysing cyanide.

The present invention allows the engineering of an integrated synthetic lixiviant biological system to be housed within a synthetic host (such as the cyanogenic *Chromobacterium violaceum*) for efficient precious metal recovery and toxic metal remediation of electronic waste. There may be up to four main components/modules in the design and engineering of the synthetic host: 1) synthetic cyanogenesis; 2) synthetic

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metal recovery; 3) synthetic cyanolysis; and 4) synthetic circuits for lixiviant biology. This invention enables synthetic circuits for lixiviant biology to be constructed.

In this invention, the inventors have designed and constructed tools to remove excess cyanide from the environment, providing industry with sustainable gold cyanidation processes. In addition, the inventors have designed and constructed genome editing tools for *Chromobacterium violaceum* for the construction of synthetic *C. violaceum* to recover precious and toxic metals from electronic waste. Further, the inventors have designed and constructed tools to reduce ionic gold ( $\text{Au}^{3+}$ ) and/or silver ( $\text{Ag}^+$ ) back to the elemental state (Au) and (Ag) as gold and silver nanoparticles, respectively, providing industry with an alternative to conventional recovery steps involving electrolysis.

According to a first aspect, the present invention provides an isolated genetically engineered bacterium, wherein the bacterium has been transformed by at least one polynucleotide molecule; the at least one polynucleotide molecule comprising a heterologous mercury(II) reductase (*MerA*) gene, operably linked to at least one promoter, and comprising at least one mutation which renders the gene product capable of reducing ionic metal to elemental metal as metal nanoparticles.

Another aspect provides an isolated genetically engineered bacterium, wherein the bacterium has been transformed by at least one polynucleotide molecule; the at least one polynucleotide molecule comprising a heterologous hydrogen cyanide synthase gene and a heterologous 3-phosphoglycerate dehydrogenase mutant gene operably linked to at least one promoter.

In some embodiments the isolated genetically engineered bacterium further comprises at least one polynucleotide molecule comprising, in order from N-terminus to C-terminus of the recombinant DNA molecule;

(i) a *goIS* transcriptional activator gene operably linked to a constitutive promoter, and a *ph1F* repressor gene operably linked to a  $P_{goITS}$  or  $P_{goIB}$  promoter;

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(ii) a promoter activated by CviR and an operator of PhIF, and

(iii) one or more of said heterologous hydrogen cyanide synthase gene and said heterologous 3-phosphoglycerate dehydrogenase mutant gene operably linked to the CviR-activated promoter.

5 Another aspect provides A process for recovering elemental gold or silver, as gold nanoparticles from ionic gold ( $\text{Au}^{3+}$ ) or as silver nanoparticles from ionic silver ( $\text{Ag}^+$ ), respectively, said process comprising the steps of:

a) contacting the isolated genetically engineered bacterium according to any one of claims 1 to 11 with a leachate comprising ionic gold ( $\text{Au}^{3+}$ ) and/or ionic silver ( $\text{Ag}^+$ );  
10 and

b) recovering the elemental gold and/or silver nanoparticles from the leachate.

According to another preferred embodiment, the present invention provides a method for producing an isolated genetically engineered bacterium, wherein the bacterium has been transformed by at least one polynucleotide molecule; the at least  
15 one polynucleotide molecule comprising a heterologous mercury(II) reductase (*MerA*) gene, operably linked to at least one promoter, and comprising one or more mutations which renders the gene product capable of reducing ionic gold ( $\text{Au}^{3+}$ ) to elemental gold as gold nanoparticles, or ionic silver ( $\text{Ag}^+$ ) which is reduced to elemental silver as silver nanoparticles, said method comprising the steps:

20 a) performing error-prone PCR on a gene encoding mercury(II) reductase (*MerA*);

i) transforming at least one bacterium with the products of said PCR;

ii) selecting transformants that grow on a media comprising  $\text{Au}^{3+}$  and/or  $\text{Ag}^+$ ; or

b) performing multiple site-saturated mutagenesis by overlap-extension PCR on a gene encoding mercury(II) reductase (*MerA*);

25 i) transforming at least one bacterium with the products of said PCR;

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ii) selecting transformants that grow on a media comprising  $\text{Au}^{3+}$  and/or  $\text{Ag}^{+}$ .

It would be understood that other forms of gold could be used on the growth media, such as  $\text{AuCl}$ .

Another aspect provides an isolated genetically engineered bacterium, wherein  
5 the bacterium has been transformed by at least one polynucleotide molecule; the at least one polynucleotide molecule comprising a heterologous nitrilase gene, the product of which causes cyanolysis of hydrogen cyanide, operably linked to at least one promoter.

In some embodiments the at least one polynucleotide molecule further comprises a heterologous formate dehydrogenase gene, a heterologous glutamate dehydrogenase  
10 gene and a heterologous phosphoenolpyruvate carboxylase gene operably linked to at least one promoter.

Another aspect provides a process of synthetic cyanide lixiviant production, said process comprising; contacting at least one recombinant cyanogenic bacterium with glycine, wherein the at least one bacterium is engineered to express a heterologous  
15 hydrogen cyanide synthase (*hcnABC*) gene and a heterologous 3-phosphoglycerate dehydrogenase mutant (*serA*) gene by linkage to at least one promoter. An example is shown in Figure 2A.

In some embodiments the recombinant cyanogenic bacterium further comprises at least one polynucleotide molecule comprising, in order from N-terminus to C-terminus  
20 of the recombinant DNA molecule;

(i) a *goIS* transcriptional activator gene operably linked to a constitutive promoter, and a *ph1F* repressor gene operably linked to a *P<sub>goITS</sub>* or *P<sub>goIB</sub>* promoter;

(ii) a promoter activated by CviR and an operator of PhIF, and

(iii) one or more of said heterologous hydrogen cyanide synthase gene and said  
25 heterologous 3-phosphoglycerate dehydrogenase mutant gene operably linked to the CviR-activated promoter.

According to another aspect, the present invention provides at least one isolated recombinant bacterium, capable of synthetic cyanide lixiviant production, as herein defined.

According to another aspect, the present invention provides a process of synthetic cyanolysis, said process comprising; contacting at least one recombinant cyanolytic bacterium with nitriles including cyanide present after bioleaching of electronic waste, wherein the at least one bacterium is engineered to express at least one nitrilase enzyme.

In some embodiments the the at least one recombinant cyanolytic bacterium is further engineered to express formate dehydrogenase, glutamate dehydrogenase and phosphoenolpyruvate carboxylase.

According to another aspect, the present invention provides an isolated recombinant DNA molecule comprising, in order from N-terminus to C-terminus of the recombinant DNA molecule;

- (i) a *golS* transcriptional activator gene operably linked to a constitutive promoter, and a *phlF* repressor gene operably linked to a *P<sub>golTS</sub>* or *P<sub>golB</sub>* promoter;
- (ii) a promoter activated by CviR and an operator of PhlF, and
- (iii) one or more cyanogenic genes operably linked to the CviR-activated promoter.

According to another aspect, the present invention provides use of a deactivated Cas9 comprising mutations H840A and D10A in the HNH endonuclease domain and the RuvC endonuclease domain, respectively, and an sgRNA to inhibit transcription of one or more genes in the *Chromobacterium violaceum* genome by targeting the promoter region of said one or more genes.

In some embodiments the one or more genes encode violacein purple pigment formation.

Another aspect provides an isolated recombinant DNA molecule comprising a *golTSB* operon, wherein the operon comprises in order from N-terminus to C-terminus

of the recombinant DNA molecule; *goIT* operably linked to j23119 promoter, *goIS*, *goIB* operably linked to *goB* promoter, and a reporter gene, such as *GFP*. This operon can be used for general screening for MerA gold-reducing engineered bacteria.

## 5 BRIEF DESCRIPTION OF THE FIGURES

**Figures 1A-1B:** **Fig. 1A;** Shows an engineered cyanogenic strain (SynLix 3.1) produces up to 80 mg/L of cyanide. SynLix 3.1 was engineered to express hydrogen cyanide synthase (*hcnABC*) and a mutant of 3-phosphoglycerate dehydrogenase (*serA*). **Fig. 1B;** An overview of the cyanolysis and coupling process. An engineered cyanogenic strain (SynLix 3.1) is tolerant at pH 10.0 (alkaliphilic).

**Figure 2** shows an overview of the cyanolysis and coupling process. Four variants of nitrilases from different bacteria were selected; their sequences were synthesized and subsequently cloned into their respective host cells. Nitrilase from *Pseudomonas pseudoalcaligenes* (*nit*) and *Synechocystis* sp. PCC 6803 chromosome (*SC-nit*), cyanide dihydratase from *Bacillus pumilus* (*BP-cynD*) and *Pseudomonas stutzeri* (*PS-cynD*). *Nit* contained 2 different subunit, namely *nitB* and *nitC*, were cloned into pRSF-Duet vector and expressed in *E.coli* (DE3) BL21 host cell. For the remaining three variants, they were cloned into pGM vector and subsequently integrated into *Chromobacterium violaceum* genome via Tn7 transposition system

**Figure 3** shows a schematic of a recombinant *Chromobacterium violaceum* host cell. Coupling enzymes include formate dehydrogenase, glutamate dehydrogenase and phosphoenolpyruvate carboxylase. These enzymes were cloned in pBbB8k-RFP (Broad Host range) vector and subsequently expressed in *Chromobacterium violaceum*. By coupling cyanolysis process to downstream enzymes, the by-products, carbon and nitrogen, can be recycled, making it a self-sustaining system

**Figure 4** shows a schematic representation of a deactivated Cas9 mechanism of transcriptional repression. The catalytically inactivated Cas9 is guided by sgRNA (blue) to the promoter sequence and physically inhibits RNAP from initiating transcription. The targeted 20 nucleotide protospacer (purple) in the promoter region is immediately

adjacent to the protospacer adjacent motif (PAM) sequence NGG (red) which is required for Cas9 binding to DNA.

**Figure 5** shows repression of violacein production in *C. violaceum* in dCas9. **(A)** Schematic of dCas9 circuitry. **(B)** The targeted region of the violacein operon are indicated by the three dCas9-sgRNA complex, the *vioA* promoter, *vioB* promoter and 5' region of *vioC*. **(C)** gRNA targeting the *vioA* promoter requires the lowest amount of dCas9 induction (0.01 % arabinose), gRNA targeting *vioB* promoter requires slightly higher amount of dCas9 induction (0.1 % arabinose), while gRNA targeting *vioC*, further down the transcription start site, has the same effect as both non-targeting gRNA negative controls. **(D)** Measured OD600 of the corresponding wells in (C).

**Figure 6** shows gold sensor circuitry optimization. Gold ions dose response and circuitry schematic with **(A)** original *GolTSB* operon or minimal gold sensor consisting of *GolS* transcriptional activator and **(B)**  $P_{golTS}$  or **(C)**  $P_{golB}$ .

**Figure 7** shows a circuitry schematic of the gold mutant sensor.

**Figure 8** shows the structure of *GolS*. **(A)** N-terminal and C-terminal domain of *GolS* consists of a helix-turn-helix (HTH) DNA-binding domain and a gold ion binding domain, respectively. **(B)** Predicted structure of *GolS* homodimer bound to DNA using Phyre2 (Kelley, Mezulis, Yates, Wass, & Sternberg, 2015). The residue (right arrows) at the DNA binding domain is mutated in *GolSmt1*, *GolSmt2* and *GolSmt3*. *GolSmt2* has an additional mutation (residue on top, up arrow) at gold ion binding domain. *GolSmt3* has an additional mutation (left arrow residue) at the DNA binding domain.

**Figure 9** shows the site of mutation of *GolSmt1A38I*. Single mutant at residue 38 from alanine to isoleucine (circled). Both residues are hydrophobic, isoleucine has 3 more carbons in the hydrophobic side chain than alanine. This may increase hydrophobicity and aggregation into the hydrophobic core and allow stronger binding with DNA.

**Figure 10** shows the mutation of *GolSmt2 A38Q N97D*. Double mutant at residue 38 from alanine to glutamine (circles at DNA binding domain) and at residue 97 from

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asparagine to aspartic acid (circles at ion binding domain). For the first mutation, alanine is a nonpolar neutral amino acid while glutamine is polar neutral with an amide side chain, the longer polar side chain of glutamine may have better binding to DNA. For the second mutation, asparagine is polar neutral while aspartic acid is polar acidic, the negatively charged side chain of aspartic acid may have a higher affinity to positively charged gold ion or improve dimerization.

**Figure 11** shows mutation of GolSmt3 A38K V60L. Double mutant at residue 38 from alanine to lysine (outer circles) and at residue 60 from valine to leucine (inner circles). For the first mutation, alanine is nonpolar neutral while lysine is polar basic, lysine may allow better binding to the backbone phosphate groups. For the second mutation, both valine and leucine are nonpolar neutral and leucine has one more carbon in its side chain, this may increase overall hydrophobicity of the DNA binding domain.

**Figure 12** shows an experimental workflow for mutagenesis and selection of gold sensor mutants in *C. violaceum*. Templates of mutant libraries of *goIS* transcriptional activator were created by deep scanning mutagenesis where each amino acid is replaced by 19 other amino acids. Mutant libraries were subsequently cloned and transformed into *C. violaceum*. Mutants with higher fluorescence output and sensitivity to gold ions were chosen.

**Figure 13** shows response of wild type and top four gold sensor mutants to gold ions. The response curves are plotted and fitted to the Hill equation  $Y = (B_{\max}X^n)/(K^n + X^n) + C$ .  $K$ : Activation  $[Au^{3+}]$  threshold at half maximal RFU,  $n$ : Hill coefficient,  $C$ : Baseline RFU,  $B_{\max}$ : Maximum RFU.

**Figure 14** shows a schematic of a synthetic circuitry providing a way to incorporate dynamic regulation into a new system. The quorum molecules allow autonomous switching ON of circuit while the gold sensor informs the circuitry to turn OFF when gold ions have been leached from the electronic waste.

**Figure 15** shows a schematic of reversible synchronized ON-OFF circuitry in response to absence and presence of gold ions. Circuitry is turned ON by the quorum sensor

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(middle box) and turned OFF by the gold ion sensor (left box). The reversible circuitry can be turned ON again in the absence of gold ions with dilution of the repressor. Gold ion sensor (left box) consists of the GolS transcriptional activator that is activated by gold ions for the expression of PhIF repressor. The quorum sensor (middle box) makes use of the endogenous CviR activator of the host that is induced by AHL under high cell density. Cyanogenesis output (right box) represented by RFP is thus synchronized at high cell density and inhibited in the presence of gold ions.

**Figure 16** shows schematic of batch culture of *Chromobacterium violaceum* for ON-OFF output in response to gold ions. To investigate the ON-OFF output of the circuit, small-scale batch cultures of *C. violaceum* were cultured in 300  $\mu$ L of Tris minimal media supplemented with 30  $\mu$ g/mL kanamycin in 2 mL eppendorf tubes for 24 hours at 37 °C. 1:600 dilution of the stationary phase culture was carried out for each passage with addition of 2  $\mu$ M AuCl<sub>3</sub> on alternate passages. End-point single cell fluorescence output was measured using BD Accuri C6 Flow cytometer (BD Biosciences, Singapore) with a flow rate of 14  $\mu$ L/min and a core size of 10  $\mu$ m, collecting 10 000 events for each sample. Fluorescence excitation at 561 nm and detection at 610/620 nm was taken. Cells were gated with forward scatter and side scatter. Background fluorescence of control cells without RFP was subtracted from mean fluorescence values.

**Figure 17** shows a graph of reduced basal leaky expression of circuitry with mutant gold sensors compared to wild type gold sensors through continuous cell passages in the presence or absence of gold ions. Each passage is 1:600 dilution of the previous passage in either minimal media without Au<sup>3+</sup> to turn ON the circuitry or with 2  $\mu$ M Au<sup>3+</sup> to turn OFF the circuitry.

**Figure 18** shows graphs representing distinct ON and OFF populations through three cycles of ON-OFF with GolS mutants (B- GolSmt1, C- GolSmt2, D- GolS mt3) while ON-OFF populations merged in circuitry containing wild type gold sensor (A).

**Figure 19** shows schematic of construction of biosensor. The Salmonella *gol* operon is comprised of *golT*, *golS* and *golB*, coding for P-type ATPase, Au sensor and metal binding chaperone respectively. Constitutive *E.coli* promoter J23119 ensures expression

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of Au sensor for binding of  $\text{Au}^{3+}$  ions which then binds Au-sensing promoter *golB* and drives expression of GFP.

**Figure 20** shows a graph of the biosensitivity of *golGFP* with respect to increasing concentration of  $\text{Au}^{3+}$  ( $\mu\text{M}$ ).

5 **Figure 21** shows schematic of screen to identify gold-reducing mutants.

**Figure 22** shows target sites in MerA protein for sequence modification to alter metal binding affinity and/or specificity.

**Figure 23** shows the effect of  $\text{Au}^{3+}$  on *E. coli* Rosetta (DE3) pLysS cell growth in (A) liquid broth and (B) agar plates.

10 **Figure 24** shows the  $\text{OD}_{600}$  of the wild-type MerA-expressing *E. coli* cells and all the mutants from  $\text{Au}^{3+}$  containing agar plate

**Figure 25** shows a TEM image of gold nanoparticles synthesized by MerA mutants.

**Figure 26** shows a comparison of  $\text{Au}^{3+}$  reduction kinetic parameters for mutants identified through DM selection. ● – DM mutant, ● – WT MerA. For (A) & (B) Mutants above the dotted line show improvement in  $k_{\text{cat}}/K_M$  or  $k_{\text{cat}}$  over WT MerA (A) Comparison of  $k_{\text{cat}}/K_M$  values for DM mutants (B) Comparison of  $k_{\text{cat}}$  values for DM mutants (C) Comparison of  $K_M$  values for DM mutants. Mutants below the dotted line show improvement in  $K_M$  over WT MerA (D) – Table of mutants and associated sequences.

20 **Figure 27** shows TEM images of AuNP recovered through reduction of  $\text{AuCl}_3$  via (A) WT MerA (B) DM11.

**Figure 28** shows *in vivo* reduction of  $\text{Au}^{3+}$  from  $\text{AuCl}_3$  and leachate via (A) WT MerA (B) DM11.

**Figure 29** shows a comparison of Au content before and after recovery by DM11, and at downstream processing stages.

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**Figure 30** shows TEM images of AuNP<sup>13</sup> recovered by DM11 from AuCl<sub>3</sub> and electronic waste leachate. **(A)** Truncated tetrahedron AuNP recovered from AuCl<sub>3</sub> **(B)** Truncated bi-tetrahedron AuNP recovered from AuCl<sub>3</sub> **(C)** Truncated tetrahedron AuNP recovered from leachate.

- 5 **Figure 31** shows **(A)** Gold and silver biosensor schematic in *C. violaceum* and **(B)** a graph of mutant gold sensor sensitivity to different metal ions, AuCl<sub>3</sub> (40 µM), AgNO<sub>3</sub> (10 µM), CdCl<sub>2</sub> (80 µM), ZnCl<sub>2</sub> (100 µM), HgCl<sub>2</sub> (5 µM), NiSO<sub>4</sub> (50 µM), CoCl<sub>2</sub> (120 µM), FeSO<sub>4</sub> (25 µM) and CuSO<sub>4</sub> (35 µM), in minimal media.

**Figure 32** shows dose-response curves of wild type and mutant biosensor to different  
10 metal ions in minimal media.

**Figure 33** shows an outline of a process for gold and silver biosensing in *C. violaceum* using leached metals from electronic scrap metal.

**Figure 34** shows a graph of the ability of wild type and mutant biosensor to sense precious metal ions in a mixture of metal ions leached from electronic scrap metal (ESM).

- 15 Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference but their mention in the specification does not imply that they form part of the common general knowledge.

## 20 DETAILED DESCRIPTION OF THE INVENTION

- It is envisioned that the use of engineered bacteria in processes involving cyanogenesis, reduction of leached metal ions, cyanolysis and downstream recycling of cyanide will require the use of separate strains of bacteria, harbouring separate mutant enzymes. Some strains will selectively reduce gold, whilst others will selectively reduce  
25 silver. A possible workflow would include: Strains that generate the biolixiviant to oxidise metals; strains will then reduce metals selectively, using gold- and silver-reducing

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mutants, to recover the metals; and strains will be used to bioremediate the biolixiviant by biodegrading cyanide.

Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that they are not limited to specific  
5 synthetic methods or specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

## 10 Definitions

Certain terms employed in the specification, examples and appended claims are collected here for convenience.

The terms "a" and "an" are used herein to refer to one or to more than one (i.e. at least one) of the grammatical object of the article.

15 As used herein, the term "comprising" or "including" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term "comprising" or "including" also includes "consisting of". The variations of the  
20 word "comprising", such as "comprise" and "comprises", and "including", such as "include" and "includes", have correspondingly varied meanings.

Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes— from the one particular value and/or to the other particular value. Similarly,  
25 when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the

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other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that

5 when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10" as well as "greater than or equal to 10" is also disclosed. It is also understood that the throughout the application, data is provided in a number of different

10 formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each unit between two particular

15 units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

In a first aspect of the invention there is provided an isolated genetically engineered bacterium, wherein the bacterium has been transformed by at least one polynucleotide molecule; the at least one polynucleotide molecule comprising a

20 heterologous mercury(II) reductase (*MerA*) gene, operably linked to at least one promoter, and comprising one or more mutations which renders the gene product capable of reducing ionic metal to elemental metal as metal nanoparticles.

In some embodiments the *MerA* gene comprises one or more mutations which encode amino acid substitutions, wherein the amino acid substitutions are at positions

25 selected from the group comprising V317, Y441 and C464. In other embodiments the *MerA* gene mutation is at one or more sites selected from the group comprising A323D, A323D (del $\Delta$ 324-365), A414E, G415I, E416C, L417I, I418D and A422N.

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In some embodiments said ionic metal is ionic gold ( $\text{Au}^{3+}$ ) which is reduced to elemental gold as gold nanoparticles, or ionic silver ( $\text{Ag}^+$ ) which is reduced to elemental silver as silver nanoparticles.

5 In some embodiments said isolated bacterium has reduced reductive capacity for mercury substrate when compared to a bacterium comprising a non-mutated *MerA* gene.

10 In some embodiments an isolated genetically engineered bacterium has been transformed by at least one polynucleotide molecule; the at least one polynucleotide molecule comprising a heterologous hydrogen cyanide synthase gene and a heterologous 3-phosphoglycerate dehydrogenase mutant gene operably linked to at least one promoter. In some embodiments the hydrogen cyanide synthase gene is hcnABC (SEQ ID NO: 35) and/or the 3-phosphoglycerate dehydrogenase mutant gene is serA (SEQ ID NO: 36). These genes are present to increase hydrogen cyanide production to leach precious metals from sources such as electronic waste.

15 In some embodiments the production of hydrogen cyanide is under the control of an on/off switch, comprising a gold ion sensor and a quorum sensor (for example shown in Figure 15). More particularly, the gold sensor switches off the leaching process once leached gold reaches a critical level. The off circuit responds to gold ions produced by cyanidation and comprises a 'low' constitutive promoter driving a *goIS* gene and a promoter driving a repressor gene which is downstream. An example of a minimal gold sensor comprises a *goIS* transcriptional activator gene under the control of a low constitutive promoter (e.g.  $P_{\text{Con6}}$ ; SEQ ID NO: 37) and a *ph1F* repressor gene driven by a  $P_{\text{goITS}}$  or  $P_{\text{goIB}}$  promoter to produce Ph1F repressor to block an operator of PhIF in the quorum sensor. The ON circuit of the quorum sensor responds to high cell density and comprises a promoter, which is activated by endogenous CviR (induced by AHL at high cell density), and an operator of PhIF downstream of the quorum sensor promoter. Downstream and under the regulation of the quorum sensor is a cyanogenesis gene or genes, selected from a heterologous hydrogen cyanide synthase gene and a heterologous 3-phosphoglycerate dehydrogenase mutant gene as described above.

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In some embodiments, the isolated genetically engineered bacterium comprises a heterologous hydrogen cyanide synthase gene and a heterologous 3-phosphoglycerate dehydrogenase mutant gene operably linked to an on/off circuit, wherein the off circuit is upstream of the on circuit which is upstream of the hydrogen cyanide synthase gene and 3-phosphoglycerate dehydrogenase mutant gene, wherein  
5 the off circuit comprises a heterologous *golS* gene, operably linked to a promoter, and a downstream heterologous *ph1F* repressor gene operably linked to a promoter selected from  $P_{golTS}$  and  $P_{golB}$ , wherein the on circuit comprises a promoter, which is activated by endogenous CviR, and an operator of PhIF between the CviR-activated promoter and  
10 wherein said hydrogen cyanide synthase gene and 3-phosphoglycerate dehydrogenase mutant genes are operably linked to said CviR-activated promoter.

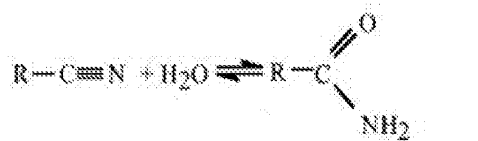
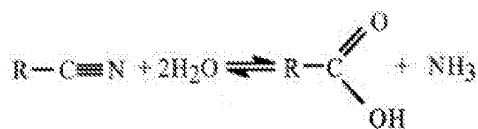
Preferably the *golS* gene is codon optimized for *C. violaceum* and the dynamic range and sensitivity is optimized by mutagenesis (for example shown in Figure 12). In some embodiments the *golS* gene is a mutant selected from GolSmt1\_A38I,  
15 GolSmt2\_A38Q&N97D, GolSmt3\_A38K&V60L and GolSmt4\_D33P.

A useful gold ion sensor comprises a golTSB operon, shown in Figure 19.

Another aspect provides an isolated recombinant DNA molecule comprising a golTSB operon, wherein the operon comprises in order from N-terminus to C-terminus of the recombinant DNA molecule; *golT* operably linked to j23119 promoter, *golS*, *golB*  
20 operably linked to *golB* promoter, and a reporter gene, such as *GFP*. This operon can be used for general screening for MerA gold-reducing engineered bacteria.

18

Nitrilases are a group of enzymes which hydrolyze nitriles to ammonia and corresponding carboxylic acid. There are two types of such cyanide-degrading enzymes.



The first, cyanide dihydratases, comprise a group of bacterial enzymes. These enzymes behave as true nitrilases, converting cyanide directly to formate and ammonia (upper scheme). On the other hand, cyanide hydratases which are of fungal origin hydrolyze cyanide to formamide (lower scheme). These hydrolytic enzymes do not require additional cofactors or substrates and catalyze over a wide substrate concentration, making them good candidates for bioremediation of cyanide.

In some embodiments an isolated genetically engineered bacterium may comprise a heterologous nitrilase gene operably linked to at least one promoter. The nitrilase gene causes cyanolysis of hydrogen cyanide. In some embodiments the heterologous nitrilase gene encodes an enzyme selected from the group comprising cyanide dehydratase and cyanide hydratase. In some embodiments the at least one nitrilase enzyme is derived from at least one bacterial species selected from the group comprising *Pseudomonas pseudoalcaligenes* (nit), *Synechocystis* sp. PCC 6803 chromosome (SC-nit), cyanide dihydratase from *Bacillus pumilus* (BP-cynD) and *Pseudomonas stutzeri* (PS-cynD). Apart from being part of an integrated workflow, the cyanolytic strains for bioremediation may be used by themselves, or as containment for cyanide-heavy industries.

In some embodiments the isolated genetically engineered bacterium further comprises a heterologous formate dehydrogenase gene, a heterologous glutamate dehydrogenase gene and a heterologous phosphoenolpyruvate carboxylase gene operably linked to at least one promoter. By coupling the cyanolysis process to downstream enzymes, formate dehydrogenase, glutamate dehydrogenase and phosphoenolpyruvate carboxylase, the by-products, carbon and nitrogen, can be recycled, making it a self-sustaining system (Fig. 3).

In some embodiments the isolated genetically engineered bacterium comprises a heterologous nitrilase gene, a heterologous formate dehydrogenase gene, a heterologous glutamate dehydrogenase gene and a heterologous phosphoenolpyruvate carboxylase gene operably linked to at least one promoter.

In some embodiments the bacterium is selected from the group comprising *Chromobacterium violaceum*, *Pseudomonas fluorescens*, *P. aeruginosa* and *Eschericia coli*.

In some embodiments the bacterium is stable at pH 10.

A second aspect of the invention provides a process for recovering elemental gold or silver, as gold nanoparticles from ionic gold ( $\text{Au}^{3+}$ ) or as silver nanoparticles from ionic silver ( $\text{Ag}^+$ ), respectively, said process comprising the steps of:

a) contacting the isolated genetically engineered bacterium according to any aspect of the invention with a leachate comprising ionic gold ( $\text{Au}^{3+}$ ) and/or ionic silver ( $\text{Ag}^+$ ); and

b) recovering the elemental gold and/or silver nanoparticles from the leachate.

In some embodiments the said contact is performed in alkaline conditions.

In some embodiments the contacting is performed at a pH of at least about 10.

A third aspect of the invention provides a method for producing an isolated bacterium comprising at least one polynucleotide molecule; the at least one

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polynucleotide molecule comprising a heterologous mercury(II) reductase (*MerA*) gene, operably linked to at least one promoter, and comprising one or more mutations which renders the gene product capable of reducing ionic metal to elemental metal as metal nanoparticles, said method comprising the steps:

- 5           a) performing error-prone PCR on a gene encoding mercury(II) reductase (*MerA*);
  - i) transforming at least one bacterium with the products of said PCR;
  - ii) selecting transformants that grow on a media comprising ionic metal; or
- b) performing multiple site-saturated mutagenesis by overlap-extension PCR on a gene encoding mercury(II) reductase (*MerA*);
- 10          i) transforming at least one bacterium with the products of said PCR;
- ii) selecting transformants that grow on a media comprising ionic metal.

In some embodiments the gene product is capable of reducing ionic gold ( $\text{Au}^{3+}$ ) and/or ionic silver ( $\text{Ag}^+$ ) to elemental gold as gold nanoparticles and/or to elemental silver as silver nanoparticles, respectively. In some embodiments in part ii) the ionic metal is selected from  $\text{AuCl}_3$  and/or  $\text{AgNO}_3$ . In some embodiments in part a) the PCR is performed with forward and reverse primers, wherein the forward primer comprises the nucleotide sequence 5'-GTGGTGGTGGTGGTGGTCTCGAGTTA-3' (SEQ ID NO: 1) and the reverse primer comprises the nucleotide sequence 5'-GATATACATATGCACCACCATCACCATCAT-3' (SEQ ID NO: 2).

20           In some embodiments in part b) the PCR is performed with primers containing NNK and/or MNN at the target sites are V317, Y441 and C464 of the *MerA* protein.

In some embodiments said selection involves at least 2 forms of selection, wherein one form comprises selection on agar plates comprising  $\text{Au}^{3+}$  and/or  $\text{Ag}^+$  and another form comprises selection in liquid culture comprising  $\text{Au}^{3+}$  and/or  $\text{Ag}^+$ .

21

A fourth aspect of the invention provides a process of synthetic cyanide  
lixiviant production, said process comprising:

contacting a recombinant cyanogenic bacterium with glycine,

wherein the bacterium comprises a heterologous hydrogen cyanide synthase  
5 gene and a heterologous 3-phosphoglycerate dehydrogenase mutant gene operably  
linked to at least one promoter.

In some embodiments the hydrogen cyanide synthase gene is *hcnABC* and/or  
the 3-phosphoglycerate dehydrogenase mutant gene is *serA*. An example is shown in  
Figure 1A, where a mutant called SynLix 3.1 was grown in LB, and cyanide production  
10 was monitored over 48 hours. Cyanide production was detected using a cyanide  
sensitive ion selective electrode (ISE).

In some embodiments the *hcnABC* and *serA* genes are under the control of  
inducible promoters. In some embodiments the isolated genetically engineered  
bacterium comprises a heterologous hydrogen cyanide synthase gene and a  
15 heterologous 3-phosphoglycerate dehydrogenase mutant gene operably linked to an  
on/off circuit, wherein the off circuit is upstream of the on circuit which is upstream of the  
hydrogen cyanide synthase gene and 3-phosphoglycerate dehydrogenase mutant gene,  
wherein the off circuit comprises a heterologous *goiS* gene, operably linked to a  
promoter, and a downstream heterologous *ph1F* repressor gene operably linked to a  
20 promoter selected from  $P_{goITS}$  and  $P_{goIB}$ , wherein the on circuit comprises a promoter,  
which is activated by endogenous CviR, and an operator of PhIF between the CviR-  
activated promoter and wherein said hydrogen cyanide synthase gene and 3-  
phosphoglycerate dehydrogenase mutant genes are operably linked to said CviR-  
activated promoter.

25 In some embodiments the recombinant cyanogenic bacterium is tolerant to a pH  
of at least about 10.

22

In some embodiments the synthetic cyanide lixiviant production is performed in a single reactor together with metal for bioleaching.

Another aspect of the invention provides at least one isolated recombinant bacterium, capable of synthetic cyanide lixiviant production, as defined in any aspect of the invention.

In some embodiments the recombinant cyanogenic bacterium is selected from the group comprising *Chromobacterium violaceum*, *Pseudomonas fluorescens*, *P. aeruginosa* and *Eschericia coli*.

Another aspect of the invention provides a process of synthetic cyanolysis, said process comprising:

a) contacting at least one recombinant cyanolytic bacterium with nitriles including cyanide present after bioleaching of electronic waste,

wherein the at least one bacterium is engineered to express at least one nitrilase enzyme.

In some embodiments the at least one nitrilase enzyme is selected from the group comprising cyanide dehydratase and cyanide hydratase.

In some embodiments the at least one recombinant cyanolytic bacterium is further engineered to express formate dehydrogenase, glutamate dehydrogenase and phosphoenolpyruvate carboxylase.

In some embodiments the at least one nitrilase enzyme is derived from at least one bacterial species selected from the group comprising *Pseudomonas pseudoalcaligenes* (nit), *Synechocystis* sp. PCC 6803 chromosome (SC-nit), cyanide dihydratase from *Bacillus pumilus* (BP-cynD) and *Pseudomonas stutzeri* (PS-cynD).

Another aspect of the invention provides an isolated recombinant DNA molecule comprising, in order from N-terminus to C-terminus of the recombinant DNA molecule;

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(i) a *go/S* transcriptional activator gene operably linked to a constitutive promoter, and a *ph1F* repressor gene operably linked to a  $P_{go/TS}$  or  $P_{go/B}$  promoter;

(ii) a promoter activated by CviR and an operator of PhIF, and

(iii) one or more cyanogenic genes operably linked to the CviR-activated promoter.

5 In some embodiments the *go/S* transcriptional activator gene is under the control of a low constitutive promoter, such as  $P_{Con6}$ .

In some embodiments the *go/S* gene is codon optimized for *C. violaceum* and the dynamic range and sensitivity is optimized by mutagenesis (for example shown in Figure 12). In some embodiments the *go/S* gene is a mutant selected from the group comprising  
10 or consisting of GolSmt1\_A38I, GolSmt2\_A38Q&N97D, GolSmt3\_A38K&V60L and GolSmt4\_D33P.

Another aspect of the invention provides use of a deactivated Cas9 comprising mutations H840A and D10A in the HNH endonuclease domain and the RuvC endonuclease domain, respectively, and an sgRNA to inhibit transcription of one or more  
15 genes in the *Chromobacterium violaceum* genome by targeting the promoter region of said one or more genes.

In some embodiments a deactivated Cas9-encoding gene is operably linked to a  $P_{araBAD}$  promoter and the RNA guide (sgRNA)-encoding gene is operably linked to a strong constitutive promoter such as J23119.

20 In some embodiments the deactivated Cas9 targets the violacein operon to prevent violacein purple pigment formation, because the pigment can complicate downstream processing steps. In some embodiments the deactivated Cas9 targets the *vioA*, *vioB* and/or *vioC* promoter, preferably all three promoters. Having now generally described the invention, the same will be more readily understood through reference to  
25 the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

**EXAMPLES**

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2012).

5

**Example 1*****Integrating nitrilases into host cells for cyanolysis***

Four variants of nitrilases from different bacteria were selected; their sequences were synthesized and subsequently cloned into their respective host cells. Nitrilase from  
10 *Pseudomonas pseudoalcaligenes* (nit) (SEQ ID NO: 11) and *Synechocystis* sp. PCC 6803 chromosome (SC-nit) (SEQ ID NO: 12), cyanide dihydratase from *Bacillus pumilus* (BP-cynD) (SEQ ID NO: 13) and *Pseudomonas stutzeri* (PS-cynD) (SEQ ID NO: 14). Nit contained 2 different subunits, namely nitB (SEQ ID NO: 15) and nitC (SEQ ID NO: 16), were cloned into pRSF-Duet vector and expressed in *E.coli* (DE3) BL21 host cell. For  
15 the remaining three variants, they were cloned into pGEM vector and subsequently integrated into *Chromobacterium violaceum* genome via Tn7 transposition system (Figs. 2 and 3).

Coupling enzymes include formate dehydrogenase, glutamate dehydrogenase and phosphoenolpyruvate carboxylase. These enzymes were cloned in pBbB8k-RFP  
20 (Broad Host range) vector and subsequently expressed in *Chromobacterium violaceum*. By coupling cyanolysis process to downstream enzymes, the by-products, carbon and nitrogen, can be recycled, making it a self-sustaining system (Fig. 3).

Cyanolytic strains of *Chromobacterium violaceum* were tested for their ability to remove cyanide from the extracellular environment. In the presence of 100 mg/L of  
25 potassium cyanide, the engineered cyanolytic *C. violaceum* strains were able to completely remove the exogenous cyanide within 24 hours.

**Example 2*****Development of genomic transcriptional control tool with dCas9 in Chromobacterium violaceum.***

CRISPR-Cas9 is widely adopted as a genome editing tool in many organisms. It  
5 has been used in mainly eukaryotes including mammalian, insect and yeast cells. The  
CRISPR-Cas system originates from the bacterial adaptive immune system where it  
inserts small fragments of invaded bacteriophages DNA into its host genome as memory  
for surveillance of possible future invasions of bacteriophages consisting of the same  
DNA fragments. CRISPR-Cas9 system from *Streptomyces pyogenes* consists of a Cas9  
10 endonuclease that together with an RNA guide, binds to DNA sequence homologous to  
the RNA guide and brings Cas9 endonuclease to cleave the double stranded DNA of  
invading bacteriophages. When used as a genome editing tool, the Cas9 endonuclease  
is expressed with a RNA guide consisting of a 20 nucleotide spacer complementary to  
target DNA followed by a 76 bp scaffold that contacts the Cas9 endonuclease. Upon  
15 guided to the target DNA by the 20 bp spacer of the RNA guide, Cas9 endonuclease  
causes double stranded breaks in the host chromosome. The host activates DNA repair  
mechanisms to the cleaved chromosome, resulting in insertions, deletions or  
homologous recombination of introduced DNA fragments into the DNA target site. With  
the ease and modularity of the RNA guided mechanism, the CRISPR-Cas9 genome  
20 editing method quickly became adopted in many organisms.

CRISPR-Cas has been adapted for many other applications. Due to the ease and  
specificity of DNA sequence recognition by RNA, many applications and variations of  
Cas9 have been applied for targeting of specific genomic locus. CRISPRi (CRISPR-  
interference) and CRISPRa (CRISPR-activation) that target gene transcription are two  
25 additions to the CRISPR/Cas genetic tools. CRISPRi (Fig. 4) is mediated by a  
deactivated Cas9 (dCas) (SEQ ID NO: 17), which has mutations H840A and D10A in the  
HNH endonuclease domain and the RuvC endonuclease domain respectively, that  
inhibits the transcription of the gene via steric hindrance of RNA polymerase binding to  
the promoter region. CRISPRa is achieved via a fusion protein between the dCas and

an activator that recruits the RNA polymerase, enhancing the transcription of the gene. Although CRISPRa has been shown to increase expression of target genes by 3-fold [Bikard, D. et al., *Nucleic Acids Res*, 41(15), 7429-7437 (2013)], CRISPRi has been applied more widely in genus belonging to *Escherichia*, *Pseudomonas*,  
5 *Mycobacterium*, *Corynebacterium*, *Clostridium* and *Bacillus*, inhibiting gene expression up to 300-fold [Cho, S. et al., *Int J Mol Sci*, 19(4). doi:10.3390/ijms19041089 (2018); Qi et al., 2013). CRISPRi tool will be explored in *C. violaceum* for transcriptional inhibition of chromosomal gene expression.

The CRISPR/Cas genome editing tool has been a powerful tool for eukaryotic  
10 cells, however, the use of CRISPR/Cas is more limited in prokaryotes due to the lethality of double stranded DNA cuts in prokaryotes. The DNA repair mechanism in prokaryotes is not sufficient to rescue the cells, resulting in lethality of bacteria when CRISPR/Cas is employed.

However, the use of the deactivated Cas9 (dCas9), with D10A mutation and  
15 H840A mutation in the catalytic domain, has been adapted to block transcription of target genes (Fig. 5). In this study, dCas9 is used to target the promoter region of the violacein operon, preventing the formation of the violacein purple pigment (Fig. 5B). The repression is the most effective when the first gene (*vioA*) promoter (SEQ ID NO: 18) is targeted, requiring only 0.01 % of arabinose. This is followed by *vioB* promoter (SEQ ID  
20 NO: 19), which requires a higher amount of 0.1 % arabinose induction of dCas9 expression for violacein repression. Inhibition at further downstream of the operon, *vioC* (SEQ ID NO: 20), exerts almost no effect on violacein transcription repression. The slight drop in OD should not be due to metabolic burden of expression of dCas. One of the  
25 main reasons could be due to overlap of the absorbance spectra of violacein, with its maximum absorbance at OD<sub>570</sub> [Swem, L.R. et al., *Mol Cell*, 35(2), 143-153 (2009)]. Another reason in the slight drop of optical density could be due to the clumping of cells when violacein pigment is not fully repressed (Fig. 5C).

Chromosomal transcriptional control of *C. violaceum* has not been reported to date. The control of endogenous genes will be useful for metabolic engineering,

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especially in the control of metabolic flux, providing fast and efficient knockdown of gene expression. As multiplexing is straightforward, many genes can be knocked down at once efficiently and quickly [Cobb, R.E. et al., *ACS Synth Biol*, 4(6), 723-728 (2014); Cress et al., 2015). Furthermore, dCas9 can be fused to other proteins including cytidine deaminases or adenine deaminases for single nucleotide mutations [Arazoe, T. et al., *Biotechnology journal*, 13(9): e1700596 (2018); Komor, Kim, Packer, Zuris, & Liu, 2016), providing tremendous opportunity for the expansion of the *C. violaceum* genetic toolbox.

### ***Cloning and expression of dCas for Chromobacterium violaceum***

The two catalytic mutations D10A and H840A were introduced in Cas9 via mutational primers and Gibson assembly. dCas was subsequently cloned under the control of the  $P_{araBAD}$  (SEQ ID NO: 21). A strong constitutive promoter, J23119 (SEQ ID NO: 22), was used to drive expression of sgRNA targeting *vioA* promoter, *vioB* promoter, 5' end of *vioC* and two non-targeting sequences (Fig. 5A). Overnight cultures of *C. violaceum* were diluted 1:100 into 96-deep well block (Nunc, Denmark) and dCas was induced by adding 0.01 % arabinose, 0.1 % arabinose, or 1 % arabinose or no induction as control. Cultures were grown at 37 °C, 280 rpm overnight before the cultures were transferred to 96-deep well plate for visualization of violacein production and OD600 measurement.

### **Example 3**

#### **20 Construction of a gold sensor in *Chromobacterium violaceum* from a natural gold operon**

An important sensor for a gold bioleaching circuitry is the gold sensor. The amount of gold leached will be able to provide dynamic feedback to the circuitry and stop the leaching process whenever the gold ions concentration reaches a critical level. The only gold biosensor demonstrated so far is the goITSB operon (SEQ ID NO: 23) from *Salmonella enterica* serovar Typhimurium str. LT2. It contains GoIS (SEQ ID NO: 24), shown to be the only MerR family transcriptional regulator reported to be able to distinguish gold ion from copper and silver ions [Cerminati, S. et al., *Biotechnol Bioeng*,

108(11), 2553-2560 (2011)]. While this gold biosensor has only been demonstrated in *E. coli* and *S. enterica* previously, it is optimized in *C. violaceum* for the first time in this report to achieve a large dynamic range.

The entire *golTSB* operon is first cloned upstream of the fluorescent protein  
5 output for the characterization of the gold sensor. However, the fluorescence output has a low dynamic range of output across the concentrations of  $\text{Au}^{3+}$  from 0.001  $\mu\text{M}$  to 10  $\mu\text{M}$  (Fig. 6A). This low dynamic range is due to the high leaky expression of 14842 RFU in the absence of  $\text{Au}^{3+}$  (Fig. 6A). In addition, there is a huge drop in cell density at high concentrations of  $\text{Au}^{3+}$  from 1  $\mu\text{M}$  onwards. The toxicity to the cell could be due to the  
10 overexpression of GolT, a transmembrane efflux P-type ATPase, which may disrupt the integrity of the cell membrane if overexpressed. This low dynamic range and cell toxicity of the gold sensor in *C. violaceum* led to subsequent optimizations being carried out in the *golTSB* operon.

In order to reduce the leaky activation due to GolS,  $P_{\text{golTS}}$  was replaced with a  
15 weak constitutive promoter to drive GolS expression, reducing the promoter strength and abolishing the positive feedback loop (Fig. 6B). Furthermore, GolT and GolB were removed to construct a minimal gold sensor and minimize possible detrimental effects on cell growth. The construct was subsequently tested with either  $P_{\text{golTS}}$  (SEQ ID NO: 25) or  $P_{\text{golB}}$  (SEQ ID NO: 26) driving the output fluorescent protein (Fig. 6B and C). The  
20 maximum dynamic range is 151-fold for pGolTS (min 110 RFU max 16416 RFU) and 113-fold change (min 50 RFU max 1648 RFU) for pGolB between 0 and 10  $\mu\text{M}$   $\text{Au}^{3+}$ .

There is also a higher fold change of pGolTS compared to pGolB between 0 and 1  $\mu\text{M}$   $\text{Au}^{3+}$  in which there are no observed toxicity to *C. violaceum*. There is a 38-fold change in dynamic range of gene expression for PgolB (min 50 max 1887 RFU) (Fig.  
25 6B) and a 62-fold change in dynamic range of gene expression for PgolTS (min 110 max 6737 RFU) (Fig. 6C) from 0 to 1  $\mu\text{M}$   $\text{Au}^{3+}$ . The PgolTS construct was subsequently chosen as the gold sensor due to its higher dynamic range.

### Improving dynamic range and sensitivity of gold sensor in *Chromobacterium violaceum* via mutagenesis

After optimizing the circuitry and regulation machinery for the gold sensor, deep-scanning mutagenesis is next carried out on the GolS transcriptional activator to further increase the sensitivity and dynamic range of the gold sensor. Although the GolS protein structure has not been solved, GolS belongs to the MerR protein family with a helix-turn-helix DNA binding domain and a gold ion binding domain (Fig. 8A) [Checa, S.K. et al., *Mol Microbiol*, 63(5), 1307-1318 (2007)]. Using the optimized circuitry consisting of the low constitutively expressed GolS and P<sub>golTS</sub> driving RFP (Fig. 7), GolS mutants with wide dynamic range and high sensitivity compared to GolS wild type were screened and selected (Fig. 12). Four mutants were selected and characterized; GolSmt1 (A38I); GolSmt2 (A38Q and N97D); GolSmt3 (A38K and V60L); and GolSmt4 (D33P). The structure of wild type GolS and mutants GolSmt1, GolSmt2 and GolSmt3 are shown in Figures 8 to 11, respectively.

The gold sensor mutants have increased sensitivity and dynamic range than the wild type gold sensors reported so far (Table 1). The  $B_{\max}$  values increased with the largest fold change of 3.5-fold of the wild type  $B_{\max}$  value. The other parameters including  $K$ ,  $n$  and  $C$  did not vary more than 2-fold, indicating that the other characteristics remain relatively similar.

**Table 1:** Parameter values from fitted response curves of gold sensor mutants.

|                 | $K$ ( $\mu$ M)  | $N$             | $B_{\max}$       | $C$          | $R^2$ | SEQ ID |
|-----------------|-----------------|-----------------|------------------|--------------|-------|--------|
| <b>GolS-mt1</b> | 2.25 $\pm$ 0.19 | 1.07 $\pm$ 0.07 | 45088 $\pm$ 1672 | 51 $\pm$ 225 | 0.998 | 27     |
| <b>GolS-mt2</b> | 1.36 $\pm$ 0.15 | 1.12 $\pm$ 0.10 | 36074 $\pm$ 1611 | 54 $\pm$ 370 | 0.996 | 28     |
| <b>GolS-mt3</b> | 1.48 $\pm$ 0.14 | 1.23 $\pm$ 0.11 | 41388 $\pm$ 1646 | 97 $\pm$ 392 | 0.996 | 29     |
| <b>GolS-mt4</b> | 4.05 $\pm$ 0.87 | 1.15 $\pm$ 0.13 | 43451 $\pm$ 4356 | 78 $\pm$ 234 | 0.998 | 30     |

|                |             |             |              |          |       |    |
|----------------|-------------|-------------|--------------|----------|-------|----|
| 30             |             |             |              |          |       |    |
| <b>GolS-WT</b> | 5.51 ± 3.33 | 1.15 ± 0.29 | 12811 ± 3825 | 47 ± 111 | 0.994 | 24 |

Sensitivity is a key parameter of sensors that determines its functionality in the system. The results obtained indicate that the gold sensor mutants are at least twice as sensitive to gold ions as the wild type gold sensor. There is a detectable fold change of at least three-fold induction at 8 nM gold ions for the gold sensor mutants while there was no detectable fold change for the wild type gold sensor (Table 2).

**Table 2:** Fold changes in output between ON and OFF state with increasing concentrations of Au<sup>3+</sup> for the wild type and the top three gold sensor mutants.

| Induction<br>(Au <sup>3+</sup> ) [μM] | Fold change |         |         |     |
|---------------------------------------|-------------|---------|---------|-----|
|                                       | GolSmt1     | GolSmt2 | GolSmt3 | WT  |
| 0                                     | 1           | 1       | 1       | 1   |
| 0.0032                                | 1           | 1       | 1       | 1   |
| 0.016                                 | 3           | 5       | 4       | 1   |
| 0.08                                  | 11          | 29      | 26      | 3   |
| 0.4                                   | 53          | 132     | 128     | 11  |
| 2                                     | 212         | 398     | 453     | 53  |
| 10                                    | 648         | 591     | 697     | 148 |

This value is more sensitive than the reported 33 nM gold ions with 2.3-fold induction as threshold for detection in an *E. coli* biosensor [Cermiati, S. et al., *Biotechnol Bioeng*, 108(11), 2553-2560 (2011)]. Gold sensor mutants achieved more than 10-fold change in output at just 80 nM Au<sup>3+</sup> while the wild type gold sensor has only 3-fold

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induction at that concentration of gold ions with sensitivity of gold ions at 4.5 RFU nM<sup>-1</sup>. There are also low leaky expression of the OFF state in both wild type and mutant gold sensors. The OFF states are low with all outputs below 100 RFU. (GoISmt1- 51 RFU, GoISmt2- 54 RFU, GoISmt3 – 97 RFU, WT – 47 RFU). The gold sensor mutants are also  
 5 able to produce higher maximum outputs than wild type gold sensor before toxic levels of gold ions are reached with more than 100-fold change (GoISmt1 – 45 088 RFU, GoISmt2 – 36 074 RFU, GoISmt3- 41 388 RFU, WT – 12 811 RFU).

All the mutants are mutated in the helix-turn-helix DNA binding domain (left and right arrows, Fig. 8B), suggesting that the increased output of the transcriptional activator  
 10 could be due to better binding to the promoter region and the subsequent activation of the promoter region, thereby leading to the higher maximum output of the gold sensor mutants (Fig. 13). The activator mutants also have increased affinity to gold ion binding, which is supported by the shift of the gold response function to the left (Fig. 13). At the same time, no leaky basal expression was found, suggesting that the mutations did not  
 15 increase activation of the non-induced activator. In conclusion, the increased transcriptional output, increased affinity to gold ions, and tight expression of the gold sensing transcriptional activator mutants could serve as useful tools for future gold ion sensing applications.

### ***Mutagenesis of gold sensor***

20 Libraries of *goIS* mutants were created by QuikScan-19 and built using the QuikChange-HT kit (Agilent Technologies, Santa Clara). Each amino acid is iteratively replaced with all 19 other amino acids, resulting in 19 mutagenic custom oligos for each amino acid in GoIS to be used in the QuikChange reaction. For the 154 aa GoIS, excluding the first amino acid methionine, there is a total of 153 x 19 = 2907 possible  
 25 single amino acid mutants to be generated. A total of 6 libraries spanning approximately 25 amino acids mutational regions each were generated across the protein according to manufacturer's protocol. Each library was subsequently transformed into competent *C. violaceum* and plated onto Tris minimal media 1.5% bacteriological agar with 30 µg/mL kanamycin and 2 µM AuCl<sub>3</sub>. Colonies were selected for higher RFP output compared to

wild type GolS by observation under blue light and inoculated into 96-well plates for growth and subsequent fluorescence quantification with BioTek Synergy H1 microplate reader.

#### ***Fluorescence measurement for quorum and gold sensor characterization***

5 For gold sensor characterization, cells were grown in 96 deep well block (Nunc, Denmark) at 37 °C overnight from frozen stocks in LB media. Cells were diluted 1:200 in 96-well plates (Costar, Kennebunk) with 0.001  $\mu$ M to 10  $\mu$ M gold (III) chloride (Sigma Aldrich) in Tris minimal media (pH 7.5) containing 80 mM NaCl, 50 mM Tris, 22 mM glucose, 20 mM KCl, 20 mM  $\text{NH}_4\text{Cl}$ , 3 mM  $\text{Na}_2\text{SO}_4$ , 1 mM thiamine hydrochloride, 0.5 g/L yeast extract, 1 mM  $\text{MgCl}_2$ , 0.65 mM  $\text{Na}_2\text{HPO}_4$  and 0.1 mM  $\text{CaCl}_2$ .  
10

Diluted cells were subsequently grown in BioTek Synergy H1 microplate reader at 37 °C overnight. Red fluorescence was measured with excitation at 530 nm, emission at 600 nm (gain 50) every 10 minutes for 14-20 hours. Optical density was measured by absorbance at 600 nm.  $\text{OD}_{600}$  and fluorescence values were blanked with media only  
15 without cells.

#### **Example 4**

##### ***Incorporation of dynamic regulation and biosensors via synthetic circuitry for a robust microbial cell factory***

In a gold bioleaching microbial cell factory, the two main constituents are, gold,  
20 which is the element of interest, and cyanide, which is the leachant needed to oxidize the solid gold into aqueous gold ions. This can pose a challenge to the living microbial cell factory as both gold ions and cyanide are highly toxic to microorganisms. Gold ions toxicity arise from the accumulation of  $\text{Au(I)}\text{-S}$  complexes which induces oxidative stress [Reith, F. et al., *Proc Natl Acad Sci U S A*, 106(42), 17757-17762 (2009)] while cyanide  
25 inhibits the respiration process by binding to metals in the cytochrome oxidase [Knowles, C.J. *Bacteriol Rev*, 40(3), 652-680 (1976); Knowles, C.J. & Bunch, A.W. *Adv Microb Physiol*, 27, 73-111 (1986)]. Gold ions are shown to be toxic to bacteria at concentration

as low as 0.35  $\mu\text{M}$  [Shareena Dasari, T.P. et al., *Biochem Pharmacol (Los Angel)*, 4(6), 199 (2015)] while cyanide toxicity to bacteria is as low as 0.4  $\mu\text{M}$  [Liu, W. et al., *Chinese Journal of Chemistry*, 25(2), 203-207 (2007)]. Although many cyanide detoxifying mechanisms [Brysk, M. M. et al., *J Bacteriol*, 97(1), 322-327 (1969); Brysk, M.M. & Ressler, *Journal of Biological Chemistry*, 245(5), 1156-1160 (1970); Ressler, C. et al., *Biochemistry*, 12(26), 5369-5377 (1973)] in *C. violaceum* are present to protect itself from its own cyanide production, gold ions toxicity will be a new pressing challenge for the uninitiated *C. violaceum* during the gold bioleaching process. Hence, a dynamic ON-OFF circuit to switch ON the production of cyanide only at high cell density and to switch OFF the circuit upon sensing of toxic gold ions was constructed (Fig. 14).

The addition of the endogenous quorum sensing system into the circuitry will ensure that there is activation of the output only when the cell density is sufficiently high (Fig. 15). As the gold sensor which initiates the synthetic circuitry's negative feedback loop is an activator, a repressor has to be introduced downstream to mediate the repression. A characterized strong repressor of the TetR family that mediates up to 193-fold change in gene expression, PhIF (SEQ ID NO: 31) [Stanton, B.C. et al., *Nat Chem Biol*, 10(2), 99-105 (2014)], is added downstream of the gold sensor for transcriptional repression of the output. The operator of PhIF (SEQ ID NO: 32) is added downstream of the quorum sensing promoter for physical inhibition of RNAP in the transcription process (Figure 15). The negative feedback loop provided by the gold sensor will grant real-time feedback on the bioleaching process and inhibit it when the cyanogenesis process has resulted in a high concentration of gold ions through cyanidation. Once it is expressed, the repressor will subsequently bind to the promoter region of the cyanogenesis gene. This means that no further expression of the bioleaching enzymes will occur when high concentration of gold ions is detected.

The circuitry is tested in continuous batch cultures with 1:600 dilution from the previous passage. The cultures are either grown in the presence of 2  $\mu\text{M}$   $\text{Au}^{3+}$  to turn OFF the circuitry or in the absence of gold ions to turn ON the circuitry (Fig. 16). 2  $\mu\text{M}$  of gold ions is used to induce the OFF state as it had resulted in the highest repression before gold ion toxicity was observed at 10  $\mu\text{M}$  in our study. However, turning OFF the

circuitry means that there should be low or no leaky expression at all. The basal expression level of the circuitry containing the wild type gold sensor in the OFF state increases over the cycles. OFF output increased from 245 RFU in the first cycle to 765 RFU and 1047 RFU for the second and third cycle respectively (Fig. 17). This is in contrast to the circuitry with the gold sensor mutants that have OFF state outputs consistently maintained below 250 RFU for all three cycles (Fig. 17).

The reduced basal expression of the GoIS mutants resulted in increased dynamic range of expression through continuous cell cultures. While the dynamic range for WT gold sensor dropped from 15-fold in the first cycle to only 5-fold and 4-fold dynamic range in the second and third cycle respectively, the circuitry containing GoIS mutants maintained dynamic range above 10-fold for all three cycles (Fig. 17). This maintenance of high dynamic range in the circuitry containing the GoIS mutants will give robustness and increased functionality of the gold leaching microbial cell factor to undergo multiple ON-OFF cycles.

The increased robustness of the GoIS mutant circuitry is further supported by the distinct ON-OFF populations maintained through continuous cycles as seen from the representative flow cytometer histograms (Fig. 18), where (A) is WT; (B) GoISmt1; (C) GoISmt2 and (D) GoIS mt3. The observed spreading of the OFF cell population in the WT gold sensor (Fig. 18A) suggests that the increased heterogeneity in the cell population expression is the main cause of the decrease in dynamic range of the circuitry. The stronger OFF state maintenance of the circuitry containing the gold sensor mutants can be attributed to the increased sensitivity to gold ions and the increased dynamic range of the gold sensor. The higher expression of the PhIF repressor under the same induction of 2  $\mu\text{M}$   $\text{Au}^{3+}$  can lead to better repression of the quorum sensing promoter even as the cell passages increase.

Another observation is that two of the GoIS mutants (GoISmt2, Fig. 18C and GoISmt3, Fig. 18D) have increasing population remaining in the OFF state over the three cycles, leading to slight reductions in the dynamic ranges. This could be due to the insufficient dilution of the PhIF repressor over the cycles, which results in a small

population of the cells remaining in the OFF state. Although the GoIS mutants allowed increased expression of the PhIF repressor for better maintenance of the OFF state output, it may have been too strong such that the cell dilutions of the PhIF repressor are inadequate to switch back the circuitry to the ON state. This suggests that GoISmt2 and GoISmt3 may not be as suitable as gold sensors for robust continuous ON-OFF cycles in the gold bioleaching synthetic circuitry.

The gold sensor mutants increased the robustness of the circuitry over three cycles of ON-OFF, consisting of six cell passages (Figs. 16 to 18). This contributes significantly to the development of a robust gold bioleaching microbial cell factory as the gold ions toxicity to the cells will mean that many cycles of gold leaching are needed.

### **Example 5**

#### ***Directed evolution of mercury(II) reductase toward gold reduction***

##### ***Determination of minimal inhibitory concentration of Au(III)***

Au(III) is known to be toxic to many bacterial cells due to its high affinity for thiol groups (-SH) and thus can affect many metabolically important enzymes and membrane-bound proteins. *E. coli* cells expressing MerA mutant enzyme with improved gold reduction activity could potentially exhibit more resistance to a toxic level of Au(III) supplemented in the culture media.

For the broth method, ligation mixture was transformed into *E. coli* Rosetta(DE3) pLysS competent cells, which were plated on normal LB agar plates. For broth medium, a 5 mL of Tris-buffered low-phosphate medium supplemented with the same antibiotics and different concentrations of AuCl<sub>3</sub> was inoculated with a single colony at 37°C for 24 h. OD<sub>600</sub> was then measured to compare cell growth. The lowest concentration of heavy metals that completely prevents growth was defined as MIC. It is shown in Figure 23A that cells were unable to grow at Au<sup>3+</sup> concentration greater than 140 µM.

For the plate method *E. coli* cells expressing wild-type MerA, cells were cultured, diluted and plated on Tris-buffered low-phosphate agar medium (Tris 6.06 g/L, NaCl 4.68

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g/L, KCl 1.49 g/L, NH<sub>4</sub>Cl 1.07 g/L, Na<sub>2</sub>SO<sub>4</sub> 0.43 g/L, MgCl<sub>2</sub>·6H<sub>2</sub>O 0.2 g/L, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.03 g/L, Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 0.23 g/L, glucose 5.0 g/L, yeast extract 0.5 g/L and agar 15 g/L) supplemented with 100 µg/mL of ampicillin, 34 µg/mL of chloramphenicol, 0.1 mM of IPTG and different concentrations of AuCl<sub>3</sub>, and grown at 37°C for 24 h. Results show that as Au<sup>3+</sup> concentration increased, fewer colonies were observed on the plates. Cell growth was completely inhibited as Au<sup>3+</sup> concentration reached 160 µM (Figure 23B).

The toxicity of gold is probably due to its high affinity for thiol groups (-SH) that exist in many metabolically important enzymes and membrane-bound proteins. When bound to gold ions, these proteins are no longer accessible to their native biologically relevant metal ions. The broth method exhibited a slightly lower minimal inhibitory concentration compared to the plate method. This difference could come from the different distribution pattern of Au<sup>3+</sup> ions between plates and liquid broth.

#### ***Directed evolution library construction***

The synthetic codon-optimized gene encoding mercury(II) reductase (MerA) was cloned in-frame with an N-terminal 6x His tag into the expression vector pET20b (Novagen) using restriction enzymes NdeI and XhoI. To evolve MerA with improved gold reducing activity, mutant libraries were constructed using error-prone PCR and site-saturation mutagenesis.

Error-prone PCR of MerA gene was carried out on the MerA gene (SEQ ID NO: 33; SEQ ID NO: 34) with the GeneMorph II Random Mutagenesis kit (Agilent Technologies) according to the manufacturers' protocol, with 50-100 ng of target DNA in the PCR reaction to achieve a medium mutation frequency (4.5-9 mutations/kb), which was confirmed by sequencing a few randomly picked colonies. The following primers were used: epPCR-fw: 5'-GTGGTGGTGGTGGTGCTCGAGTTA-3' (SEQ ID NO: 1) and epPCR-rv: 5'-GATATACATATGCACCACCATCACCATCAT-3' (SEQ ID NO: 2). The thermocycling program consisted of an initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min, with the last cycle followed by a 10-min extension at 72°C. The PCR product was then treated with DpnI for template degradation and purified using the QIAquick

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PCR purification kit (Qiagen). The purified DNA fragment was double digested with NdeI and XhoI, purified and ligated into the pET20b vector.

Multiple site-saturation mutagenesis was done by overlap-extension PCR using degenerate primers containing NNK at the target sites V317, Y441, C464 and C465'.

- 5 These positions are in close contact with the potential gold binding site within the active site of MerA (Fig. 22). The redox active cysteines C136 and C141 were kept unchanged to prevent possible redox activity loss. The primers used are listed in Table 3.

**Table 3:** Sequences of the primers used for site-saturation mutagenesis

| Primer     | Sequence <sup>a</sup>                             | SEQ ID |
|------------|---------------------------------------------------|--------|
| T7-fw      | 5'-TAATACGACTCACTATAGGG-3'                        | 3      |
| T7-rv      | 5'-GCTAGTTATTGCTCAGCGGTG-3'                       | 4      |
| V317NNK-fw | 5'-GATCAACCGCAGTTCNNKTATGTTGCGGCTGCC-3'           | 5      |
| V317NNK-rv | 5'-GGCAGCCGCAACATAMNNGAACTGCGGTTGATC-3'           | 6      |
| Y441NNK-fw | 5'-CGGATCAGCTGTTTCCGNNKCTGACAATGGTCTGAAG-3'       | 7      |
| Y441NNK-rv | 5'-CTTCGACCATTGTCAGMNNCGGAAACAGCTGATCCG-3'        | 8      |
| C464NNK-fw | 5'-GATGTCAAACAATTGTCGNNKNNKGCAGGCTAACTCGAGCACC-3' | 9      |
| C464NNK-rv | 5'-GGTGCTCGAGTTAGCCTGCMNNMNNCGACAATTGTTTGACATC-3' | 10     |

<sup>a</sup> Degeneracy alphabet: N = (A, T, C, G); K = (T, G); M = (A, C)

- 10 PCR reactions were performed with primer pairs T7-fw/V317NNK-rv, V317NNK-fw/Y441NNK-rv, Y441NNK-fw/C464NNK-rv and C464NNK-fw/T7-rv, to generate partially overlapping DNA fragments. The reaction mixture in a total volume of 50 µL containing 50 ng of the pET20bMerA plasmid, 500 nM of each primer and 1 x PrimeSTAR Max Premix (Clontech), was incubated with 30 cycles of 98°C for 10 s, 55°C for 5 s and
- 15 72°C for 10 s. The PCR products were purified and equimolar amount of each fragment was mixed in 1 x PrimeSTAR Max Premix for a short overlap extension reaction (5 cycles of 98°C for 10 s, 55°C for 5s, 72°C for 10 s), and 1 µL of the reaction mixture was used as the template to amplify the full length MerA mutant gene using primers epPCR-fw and epPCR-rv. The PCR product was purified, digested with NdeI and XhoI, and ligated into
- 20 the pET20b vector.

**Selection for gold tolerant mutants**

The ligation mixture from either error-prone PCR or site-saturation mutagenesis was used to transform *E. coli* Rosetta(DE3)pLysS competent cells, and the transformants were plated on Tris-buffered low-phosphate agar medium (Tris 6.06 g/L, NaCl 4.68 g/L, KCl 1.49 g/L, NH<sub>4</sub>Cl 1.07 g/L, Na<sub>2</sub>SO<sub>4</sub> 0.43 g/L, MgCl<sub>2</sub>·6H<sub>2</sub>O 0.2 g/L, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.03 g/L, Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 0.23 g/L, glucose 5.0 g/L, yeast extract 0.5 g/L and agar 15 g/L) supplemented with 100 µg/mL of ampicillin, 34 µg/mL of chloramphenicol, 0.1 mM of IPTG and 250 µM of AuCl<sub>3</sub>, and grown at 37°C for 24 h. Colonies were then picked and grown in liquid Tris-buffered low-phosphate media supplemented with 100 µg/mL of ampicillin, 34 µg/mL of chloramphenicol, 0.1 mM of IPTG and 300 µM of AuCl<sub>3</sub>. After a few rounds of selection, 10 mutants were obtained that showed substantially better cell growth at toxic level of Au<sup>3+</sup> (300 µM), where cells expressing wild-type MerA could barely grow (Fig. 24). It is worth mentioning that all the colonies that survived two selections were from the site-saturation library, indicating that this semi-rationally designed library, rather than the one generated by random mutagenesis, may contain functionally improved variants with a better chance. The improved tolerance compared to the wild type may come from two sources: 1) detoxification by MerA that enzymatically reduce Au<sup>3+</sup> ions, and 2) sequestering of Au<sup>3+</sup> by MerA binding it.

MerA mutants were produced that synthesized gold nanoparticles from AuCl<sub>3</sub> as shown in a TEM image (Fig. 25). Through our directed evolution methods, we have identified an enhanced gold-reductase, a MerA mutant V317S. This mutant has kinetic parameters listed in Table 5. The enhanced gold reductase has an increase of 67-fold in catalytic efficiency in reducing Au<sup>3+</sup> to elemental gold (Au<sup>0</sup>).

**Table 5:** Kinetic parameters of enhanced gold-reductase

| Parameter                                                           | Au <sup>3+</sup> Substrate   |                              |               |
|---------------------------------------------------------------------|------------------------------|------------------------------|---------------|
|                                                                     | Wild-type MerA               | V317S Mutant MerA            | Fold-increase |
| K <sub>M</sub> (µM)                                                 | 380 ± 140                    | 50 ± 8                       | -             |
| k <sub>cat</sub> (s <sup>-1</sup> )                                 | 6.8 ± 1.9 × 10 <sup>-3</sup> | 58 ± 2.0 × 10 <sup>-3</sup>  | 8.5           |
| k <sub>cat</sub> /K <sub>M</sub> (M <sup>-1</sup> s <sup>-1</sup> ) | 1.8 ± 1.3 × 10 <sup>1</sup>  | 1.2 ± 0.25 × 10 <sup>3</sup> | 67            |

### Further improved mutants

MerA's gold reduction capability has been established previously with a catalytic efficiency of  $9.1 \pm 3.2 \times 10^1 \text{ M}^{-1} \text{ s}^{-1}$ . A combination of directed evolution and rational design approaches was used to generate a library of improved  $\text{Au}^{3+}$  reducing MerA mutants. Over 50% of the mutants isolated showed improved activity with the highest improving mutant displaying up to 15-fold improvement in catalytic efficiency (Figure 26). Mutant DM11 (G415I) (SEQ ID NO: 48) displayed the greatest improvement in catalytic efficiency (15-fold increase) as a result of improvement in both turnover ( $k_{\text{cat}}$ ) and binding affinity ( $K_{\text{M}}$ ). The kinetic parameters of all mutants can be found in Table 6.

This improvement in catalytic efficiency is also reflected in the complexity of gold nanoparticles (AuNPs) formed after reduction has occurred. DM11 produces AuNPs of greater size and complexity as compared to MerA (Fig. 27). This improvement in catalytic efficiency is also reflected in the improved reduction and recovery of gold by DM11 from electronic waste leachate as compared to WT MerA (Fig. 28). DM11 is equally effective at recovering  $\text{Au}^{3+}$  from a solution of  $\text{AuCl}_3$  (67%) and electronic waste leachate (67%) (Fig. 29).

**Table 6:** Kinetic parameters for mutants identified through DM selection. Mutants in the dimerization region (DM10-15) and residue position 323 (DM4 and DM5) show the greatest increases in catalytic efficiency. \*(del $\Delta$ 324 - 365)

| no. | Sequence | $k_{\text{cat}}$<br>( $\times 10^{-2} \text{ s}^{-1}$ ) |       | $K_{\text{M}}$ ( $\mu\text{M}$ ) |       | $k_{\text{cat}}/K_{\text{M}}$<br>( $\times 10^1 \text{ M}^{-1} \text{ s}^{-1}$ ) |      | SEQ ID<br>NO |
|-----|----------|---------------------------------------------------------|-------|----------------------------------|-------|----------------------------------------------------------------------------------|------|--------------|
| 0   | WT MerA  | $2.7 \pm 0.3$                                           | -     | $290 \pm 85$                     | -     | $9.1 \pm 3.2$                                                                    | -    |              |
| DM1 | P49I     | $3.5 \pm 1.5$                                           | +29%  | $370 \pm 300$                    | -29%  | $9.3 \pm 5.0$                                                                    | +3%  | 38           |
| DM2 | I293F    | $4.9 \pm 2.3$                                           | +80%  | $310 \pm 300$                    | -8%   | $16 \pm 8$                                                                       | +71% | 39           |
| DM3 | T299R    | $11.7 \pm 3.7$                                          | +333% | $680 \pm 330$                    | -134% | $17 \pm 11$                                                                      | +89% | 40           |

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|             |        |            |       |           |      |           |        |    |
|-------------|--------|------------|-------|-----------|------|-----------|--------|----|
| <b>DM4</b>  | A323D  | 5.4 ± 0.6  | +102% | 61 ± 25   | +79% | 90 ± 25   | +889%  | 41 |
| <b>DM5</b>  | A323D* | 10.2 ± 1.6 | +277% | 96 ± 55   | +67% | 106 ± 29  | +1068% | 42 |
| <b>DM6</b>  | L395G  | 19.1 ± 6.3 | +607% | 440 ± 250 | -52% | 43 ± 25   | +375%  | 43 |
| <b>DM7</b>  | G406D  | 1.9 ± 0.7  | -29%  | 260 ± 210 | +10% | 7.3 ± 3.4 | -20%   | 44 |
| <b>DM8</b>  | A411Q  | 2.8 ± 0.4  | +4%   | 150 ± 57  | +48% | 18 ± 6.3  | +102%  | 45 |
| <b>DM9</b>  | A411C  | 1.1 ± 0.2  | -60%  | 72 ± 58   | +75% | 15 ± 3.5  | +65%   | 46 |
| <b>DM10</b> | A414E  | 2.4 ± 0.5  | -10%  | 87 ± 76   | +70% | 28 ± 6.9  | +207%  | 47 |
| <b>DM11</b> | G415I  | 6.8 ± 0.3  | +152% | 50 ± 11   | +83% | 135 ± 27  | +1386% | 48 |
| <b>DM12</b> | E416C  | 8.0 ± 1.9  | +195% | 230 ± 130 | +19% | 34 ± 14   | +274%  | 49 |
| <b>DM13</b> | L417I  | 4.6 ± 0.5  | +70%  | 160 ± 49  | +44% | 28 ± 11   | +211%  | 50 |
| <b>DM14</b> | I418D  | 3.3 ± 0.3  | +23%  | 52 ± 25   | +82% | 64 ± 14   | +606%  | 51 |
| <b>DM15</b> | A422N  | 3.0 ± 0.5  | +9%   | 69 ± 47   | +76% | 43 ± 10   | +375%  | 52 |
| <b>DM16</b> | L448T  | 3.8 ± 0.7  | +40%  | 410 ± 140 | -42% | 9.2 ± 5.2 | +1%    | 53 |

However, with reduction from AuCl<sub>3</sub>, AuNPs of increasing complexity can be observed, while with reduction from leachate, AuNPs of limited complexity can be observed (Fig. 30). This is likely due to the overall lower quantity of Au<sup>3+</sup> in leachate. It is believed that if this limitation can be overcome, a greater improvement in gold recovery can be observed. MerA is an essential enzyme in bacterial mercury resistant system. Based on its reaction mechanism and available crystal structure, we envisioned that it could also be engineered for efficient gold reduction. To this end, a high-throughput selection procedure was established that involves toxic agar plate selection followed by a more stringent liquid culture selection. Mutant libraries were constructed by both error-prone PCR and multiple site-saturation mutagenesis, and were subjected to this two-step selection. As a result, we have identified mutants of MerA with enhanced

gold-reduction / gold-recovery properties. This constitutes the Synthetic Metal Recovery portion of Synthetic Lixiviant Biology.

### ***Protein expression and purification***

Recombinant proteins were expressed using the T7 expression system.

5 Rosetta(DE3)pLysS cells were transformed with the plasmids and selected on LB agar plates containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. A single colony was picked into 5 mL of LB medium containing the two antibiotics and grown overnight at 37°C. A 100-fold dilution was made, and the culture was grown at 37°C until OD<sub>600</sub> reaches 0.6. Protein expression was then induced by adding isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 0.1 mM. After induction the cells were

10 grown at 16°C for a further 18 h. The cells were lysed by sonication, and the His-tagged proteins were purified from the cleared lysates on nickel-chelate columns (Qiagen). The protein samples in the eluates (500 mM imidazole, 50 mM Tris–Cl pH 7.5, 300 mM NaCl) were concentrated and dialysed against 20 mM sodium phosphate (pH 7.4) by Amicon

15 Ultra centrifugal filters (Millipore).

### ***Gold reduction assay***

The enzyme assays were carried out at 25°C in 20 mM sodium phosphate, pH 7.4, 200 µM NADPH, 100 µM AuCl<sub>3</sub>. The oxidation of NADPH was followed spectrophotometrically at 340 nm. Units of enzyme activity are defined as the amount of

20 enzyme that catalyzes the Au-dependent oxidation of 1.0 µmol of NADPH per min.

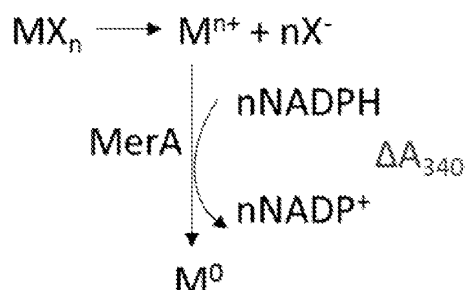
### **Example 6**

#### ***Kinetic parameters for gold reduction by MerA***

The kinetic parameters of the purified mercuric reductase enzyme (MerA) were determined for substrate AuCl<sub>3</sub> using a continuous spectrophotometric assay (Scheme

25 1).

**Scheme 1:** Assay monitoring activity of MerA via change in absorbance at 340nm. M refers to either  $\text{Hg}^{2+}$  or  $\text{Au}^{3+}$  with X then corresponding to  $\text{GSH}^-$  or  $\text{Cl}^-$ .



The reduction of  $\text{Au}^{3+}$ , the native substrate, to  $\text{Au}^0$  is coupled to the oxidation of NADPH to  $\text{NADP}^+$ . The oxidation of NADPH was observed by measuring the change of absorbance at 340 nm. A 50- $\mu\text{L}$  reaction mixture contained 100 mM PIPES (pH7.0), 400  $\mu\text{M}$  NADPH, 17.9  $\mu\text{M}$  MerA, and varying amounts of  $\text{Au}^{3+}$ . The assay was also used to observe the reduction of  $\text{Hg}^{2+}$ , MerA's native substrate, to  $\text{Hg}^0$  using substrate  $\text{Hg}(\text{GSH})_2$ .

The kinetic parameters of MerA using  $\text{Hg}(\text{GSH})_2$  and  $\text{AuCl}_3$  were determined (Table 4).

**Table 4:** Kinetic parameters of purified MerA

| Parameter                                              | Substrate                 |                                                |
|--------------------------------------------------------|---------------------------|------------------------------------------------|
|                                                        | $\text{Hg}^{2+}$          | $\text{Au}^{3+}$                               |
| $K_M$ ( $\mu\text{M}$ )                                | $96 \pm 58$               | <b><math>380 \pm 140</math></b>                |
| $k_{\text{cat}}$ ( $\text{s}^{-1}$ )                   | $15 \pm 5.1$              | <b><math>6.8 \pm 1.9 \times 10^{-3}</math></b> |
| $k_{\text{cat}}/K_M$ ( $\text{M}^{-1} \text{s}^{-1}$ ) | $1.5 \pm 0.7 \times 10^5$ | <b><math>1.8 \pm 1.3 \times 10^1</math></b>    |

With respect to mercury substrate, MerA shows a higher  $K_M$  of  $96.3 \pm 57.6 \mu\text{M}$  as compared to reported literature of  $10.7 \mu\text{M}$ . The  $k_{\text{cat}}$  value of  $14.6 \pm 5.1 \text{ s}^{-1}$  is slightly faster as compared to reported findings of  $9.43 \text{ s}^{-1}$  though, the difference is not significant. Overall catalytic efficiency ( $k_{\text{cat}}/K_M$ ) value of  $1.5 \pm 0.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , is six times lower than expected when compared to literature findings of  $8.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  (Moore, M.J., Miller, S.M., Walsh, C.T. C-Terminal Cysteines of Tn501 Mecercuric Ion Reductase (1992) *Biochemistry* 31(6):1677-85). With respect to  $\text{AuCl}_3$ , no published data is

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available. When compared to the native substrate,  $\text{Hg}(\text{GSH})_2$ , the  $k_{\text{cat}}/K_M$  using  $\text{AuCl}_3$  is four magnitudes lower. Thus, the assay is able to be used to determine MerA's reductive capabilities with regards to different substrates.

Improved gold reducing MerA mutants were identified via screening with a gold-sensing biosensor. The *goITSB* operon isolated from *Salmonella enterica* serovar typhimurium functions as a biosensor (Zammit et al., 2013) together with green fluorescent protein (GFP) as a reporter (Fig. 19). This biosensor is under the control of the GoIS regulator which is induced in the presence of  $\text{Au}^+/\text{Au}^{3+}$  complexes. When  $\text{Au}^+/\text{Au}^{3+}$  ions interact with the GoIS regulator, the GoIS regulator binds to its target promoter sequence, *goIB*. This induces a conformational change in the GoIS/*goIB* complex which promotes transcription of GFP reporter. We propose to integrate this circuit into the *E. coli* chromosome as previously shown [Cerminati, S. et al., *Biotechnol Bioeng*, 108(11), 2553-2560 (2011)], and use this *E. coli* strain for endogenous reporting of gold reducing capabilities

Biosensor was cloned into the pRSFDuet-1 vector and responsiveness to gold substrate tested. Fluorescence measurements were taken using 485\_20 and 528\_20 nm filters for excitation and emission wavelengths respectively. The final optical density at 600 nm ( $\text{OD}_{600}$ ) of each sample was also measured. Fluorescence measurements ( $F_s$ ) were normalized following the formula (Formula 1).

$$F_s = \frac{RFU^{sample}}{OD_{600}^{sample}} - \frac{RFU^{PRSF}}{OD_{600}^{PRSF}}$$

Where  $RFU^{sample}$  is the fluorescence (measured in instrument's arbitrary relative fluorescence units) and  $OD_{600}^{sample}$ , the final optic density determined for each sample obtained from the sensor bacteria, and  $RFU^{PRSF}$  and  $OD_{600}^{PRSF}$ , the same parameters determined for the strain carrying the pRSFDuet-1 vector. Induction coefficients (IC) were calculated where  $F_{\text{Au}^{3+}}$  is the normalized fluorescence value of the sensor bacteria exposed to the metal, and  $F_{\text{H}_2\text{O}}$  is the

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normalized fluorescence of the biosensor cultured without metal added (background fluorescence).

Fluorescence increased with increasing concentration of  $\text{Au}^{3+}$  concentration up to  $100\mu\text{M}$  (Fig. 20). At higher concentrations, fluorescence decreased sharply presumably due to susceptibility of *E.coli* to metal toxicity. The limits for  $\text{Au}^{3+}$  detection, i.e., the lowest and highest gold concentrations that produce a detectable increase in fluorescence compared to the background were determined to be  $100\text{nm}$  to  $100\mu\text{M}$  for the sensor. Thus, the sensor has proven to be capable of distinguishing different concentrations of  $\text{Au}^{3+}$  and will be utilized in the screen for an optimal gold reducing MerA mutant.

The screen for an optimal gold reducing MerA mutant is based on observing for a drop in fluorescence, which corresponds to a drop in  $\text{Au}^{3+}$  ions present intracellularly, hence acting as an indirect reporter for gold reducing activity (Fig. 21). In the presence of gold ions and a control plasmid, the biosensor will be induced, producing a strong fluorescence signal, but in the presence of a gold reducing MerA mutant, gold ions will be reduced, resulting in lack of fluorescence.

### Example 7

#### ***Gold biosensor in Chromobacterium violaceum is sensitive to silver ions.***

The biosensor shown in Figure 31A was tested for its sensitivity to different metal ions in minimal media. The biosensor senses the gold and silver ions but did not have high response to other metals including cadmium, zinc, mercury, nickel, cobalt, iron and copper ions (Fig. 31B). The dose response shows that the mutant biosensor is more sensitive with a larger dynamic range than the wild type biosensor to gold and silver ions (Figure 32).

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**Example 8*****Sensing of leached precious metal ions from electronic waste by *C. violaceum****

*C. violaceum* was incubated with electronic waste and cultured for 6 days to leach precious metals from electronic waste into the aqueous culture. After culturing for 6 days, the *C. violaceum* cells were centrifuged down and spent media was added to *C. violaceum* containing the biosensor used in Example 7 (Fig. 33). The transcriptional factor biosensor was activated in the spent culture media with leached ESM but not the control culture media (Fig. 34). The mutant biosensor fluorescence output was 2515 RFU while the wild type biosensor was 1101 RFU in response to the day 6th spent culture with leached ESM. ICP analysis shows that in the 6 days spent culture, there are 1.1 ppm (5.6  $\mu$ M) gold ions, 0.34 ppm (3.2  $\mu$ M) silver ions and 12.3 ppm (193.2  $\mu$ M) copper ions in the spent media. This shows that the biosensor in *C. violaceum* was activated in the presence of gold ions and silver ions from the leached electronic waste media. This will be useful when activating further synthetic circuitries module in *C. violaceum*.

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**Summary**

1. Specific to this invention, we have achieved biological reduction of metal-cyanide complexes after metal cyanidation, using the mercury reductase (Mer) system.
2. Specific to this invention, the tools will allow synthetic circuits for lixiviant biology to be constructed, and refer to the sequential and appropriate expression and activation of respective biological systems for efficient metal recovery, and include the following features:
  - a. Regulated, inducible expression of synthetic cyanogenesis modules upon addition of electronic waste to bioreactor. This enables the timely production of cyanide lixiviant for metal bioleaching.

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- 5      b. Specific, temporal expression of synthetic metal recovery modules upon detection of respective metallo-cyanide complexes. Due to the heterogeneity of electronic waste, differing concentrations of metals (and the associated metallic ions) necessitate the temporal removal of respective metals; this will be achieved through the selective expression of engineered Mer systems specific towards metal ion valency and identity.
- 10      c. Regulated, inducible expression of synthetic cyanolysis modules after metal recovery to metabolize excess cyanide lixiviant, channelling the carbon and nitrogen atoms of cyanide back into central metabolic pathways.

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**CLAIMS:**

1. An isolated genetically engineered bacterium, wherein the bacterium has been transformed by at least one polynucleotide molecule; the at least one polynucleotide molecule comprising a heterologous mercury(II) reductase (*MerA*) gene, operably linked to  
5 at least one promoter, and comprising a mutation which confers the gene product improved catalytic efficiency of reducing ionic gold to elemental gold as gold nanoparticles when compared with wild type *MerA*, wherein the mutation encodes an amino acid substitution at a position selected from the group comprising V317S, A323D, A323D (del $\Delta$ 324-365), A414E, G415I, E416C, L417I, I418D and A422N of the wild type *MerA* consisting of the amino acid  
10 sequence set forth in SEQ ID NO: 34.
2. The isolated bacterium of claim 1, wherein said at least one isolated bacterium comprises a G415I mutation which confers improved catalytic efficiency of reducing ionic gold to elemental gold as gold nanoparticles, ionic silver to elemental silver as silver nanoparticles, and reduced reductive capacity for mercury substrate when compared to a  
15 bacterium comprising a non-mutated *MerA* gene.
3. The isolated bacterium of any one of the previous claims, wherein the bacterium is selected from the group comprising *Chromobacterium violaceum*, *Pseudomonas fluorescens*, *P. aeruginosa* and *Eschericia coli* and/or wherein the bacterium is stable at pH  
10.
- 20 4. A process for recovering elemental gold, as gold nanoparticles from ionic gold ( $\text{Au}^{3+}$ ), said process comprising the steps of:
- a) contacting the isolated genetically engineered bacterium according to any one of claims 1 to 3 with a leachate comprising ionic gold ( $\text{Au}^{3+}$ ); and
- b) recovering the elemental gold nanoparticles from the leachate.
- 25 5. The process according to claim 4, wherein the said contact is performed in alkaline conditions.