Global transcriptional analysis of *Geobacter sulfurreducens* under palladium reducing conditions reveals new key cytochromes involved

Alberto Hernández-Eligio,[a]† Aurora M. Pat-Espadas,[b,d]† Leticia Vega-Alvarado,[c] Manuel Huerta-Amparán,[a] Francisco J. Cervantes,[b]# Katy Juárez[a]*

[a]Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico.

[b]División de Ciencias Ambientales, Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), San Luis Potosí, SLP, Mexico.

[c]Instituto de Ciencias Aplicadas y Tecnología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Ciudad de México, Mexico.

[d]Instituto de Geología, Universidad Nacional Autónoma de México, Hermosillo, Sonora, Mexico

†CONACYT

#Address correspondence to Francisco J Cervantes: fjcervantes@ipicyt.edu.mx.

*Address correspondence to Katy Juárez: katy@ibt.unam.mx.

Running Head: Pd(II) reduction mechanism in *Geobacter sulfurreducens*

Abstract

Geobacter sulfurreducens is capable of reducing Pd(II) to Pd(0) using acetate as electron donor; however, the biochemical and genetic mechanisms involved in this process have not been described. In this work, we carried out transcriptome profiling analysis to identify the genes involved in Pd(II) reduction in this bacterium. Our results showed that 252 genes were upregulated while 141 were downregulated during Pd(II) reduction. Among the upregulated genes, 12 were related to energy metabolism and electron transport; 50 were classified as involved in protein synthesis; 42 were associated with regulatory functions and transcription, and 47 have no homologs with known function. RT-qPCR data confirmed upregulation of genes encoding PilA, the structural protein for the electrically conductive pili, as well as *c*-type cytochromes GSU1062, GSU2513, GSU2808, GSU2934, GSU3107, OmcH, OmcM, PpcA, PpcD under Pd(II)-reducing conditions. Δ*pilA* and Δ*pilR* mutant strains showed 20% and 40% decrease in the Pd(II)-reducing capacity, respectively, compared to the wild type strain, indicating the central role of the pili in this process. RT-qPCR data collected during Pd(II) reduction also confirmed downregulation of the *omcB, omcC, omcZ,* and *omcS* genes, which have been shown to be involved in Fe(III) reduction and electrodes. Based on these results, we propose mechanisms involved in Pd(II) reduction by *G. sulfurreducens*.

Importance

Geobacter sulfurreducens is a versatile microorganism, known for its ability to reduce a wide range of environmentally relevant metals. It has been reported that this bacterium

synthesizes palladium nanoparticles successfully. Yet, the biochemical and genetic mechanisms involved in this process had not been described previously. By using a transcriptome profile analysis to identify genes implicated in this process and genetic and physiological data, we propose a model for the biological reduction of Pd(II) by *G. sulfurreducens*. Moreover, the study also revealed the microbial reduction of Pd(II) coupled to growth, which shows not only unexpected ideas about its complex metabolism, but some key cytochromes involved in this pathway, which have not been previously associated with other metal reducing process for this bacterium. In brief the present work contributes to a better understanding of the biochemical and genetic mechanisms involved in Pd(II) reduction and put forward novel insights about *G. sulfurreducens* metabolism.

Introduction

In the last two decades, the alternative of using bioreductive deposition of precious metals, such as the platinum-group (PGMs; e.g Pt, Pd, Rh), for their recovery has been widely explored. Special interest has been focused on palladium (Pd) due to its high value and extensive use as catalyst. Fe(III) reducing bacteria (IRB), such as *Shewanella oneidensis* (1) and *Geobacter sulfurreducens* (2), as well as sulfate reducing bacteria (SRB), such as *Desulfovibrio desulfuricans* (3), have extensively been explored for this purpose. Despite the great demand for developing efficient microbial processes to recover this valued element, the mechanisms involved in Pd(II) reduction and subsequent deposition of Pd(0) are poorly understood.

Extracellular formation of Pd(0) nanoparticles (NP's) has been reported in *G. sulfurreducens* (2). Moreover, differences regarding location of deposited NP's, depending on the strain, have been documented (3). These findings could be related to specific

mechanisms used by each strain, as well as to experimental conditions prevailing; however, further studies are required to fully elucidate the mechanisms involved.

Geobacter species are abundant in nature and have the ability to perform extracellular electron transfer (EET) to reduce a broad array of heavy metals, such as Fe(III), Mn(IV), U(VI), Co(III) and Ag(I), among others (4, 5, 6, 7). Regarding Pd(II) reduction, using acetate as electron donor, *G. sulfurreducens* has been used to reduce up to 25 mg Pd(II)/L with the concomitant deposition of Pd(0) NP's (2). It has been proposed that the electron transfer reported for iron reduction could also be involved in Pd(II) reduction (8). Nevertheless, the genome of *G. sulfurreducens* has 111 predicted c-type cytochromes (9, 10), hence it is likely that other cytochromes, as well as conductive pili, could play a role in Pd(II) reduction (11, 12). This is supported by the observation that some proteins or cytochromes are specifically required to achieve the reduction of certain metal species, as it has been reported for soluble Fe(III), Fe(III) oxides and U(VI) (9, 13, 14).

The purpose of this study was to elucidate the mechanisms involved in Pd(II) reduction by *G. sulfurreducens*, based on global transcriptome analysis using RNA sequencing analysis. Quantitative reverse transcription PCR, genetic and physiological tests were also performed to identify genes involved in Pd(II) reduction. The Pd(II) reduction pathways in *G. sulfurreducens* were proposed based on the obtained results.

Materials and Methods

Culture procedures

Bacterial strains and oligonucleotides used in this study are listed in Table 1. *G. sulfurreducens* PCA was grown anaerobically at 30 ^{o}C in NBAF medium, supplemented

with acetate and fumarate; these culture conditions were referred to as "Non-Pd(II)-reducing conditions" (15). For experiments conducted under "Pd(II)-reducing" conditions, late logarithmic phase cultures of *G. sulfurreducens* were used. The protocol consisted of harvesting cells by centrifugation at 9,000 *g* for 20 min and washing with a sterilized, osmotically balanced buffer. The reaction buffer composition was as follows (in grams per liter): $NaHCO_3$, 2.5; NH_4Cl, 0.25; $NaH_2PO_4 \cdot H_2O$, 0.006; and KCl, 0.1. Reduction experiments were performed in 120-ml glass serum bottles capped with rubber stoppers including 100 ml of anaerobic basal medium, flushed with N_2/CO_2 (80:20, v/v). Cell suspensions, as well as acetate and Na_2PdC_4 (Sigma-Aldrich) stock solutions were added to yield a concentration of 800 mg l^{-1} cell dry weight (CDW), 5 mM and 25 mg l^{-1}, respectively (2, 8). RNA Later (Ambion) was added to cultures and cells for harvesting cells. Bacterial pellets were flash frozen and stored at -70 ^{o}C.

RNA extraction

G. sulfurreducens cells from both "Pd(II) reduction" and "Non-Pd(II) reduction" conditions were used for RNA-Seq and RT-qPCR analyses. All experiments were performed in duplicates. For each biological sample, total RNA samples were extracted using the RNeasy mini kit (Qiagen), then they were examined with an Agilent 2100 Bioanalyzer and quantified using NanoDrop 200c (Thermo Scientific).

RNA-Seq and data analysis

RNA-Seq was performed using RNA samples extracted from two independently grown cultures with and without Pd(II). Illumina sequencing was performed at the USMI (Unidad de Secuenciación Masiva, UNAM, Mexico). Briefly, after removing residual DNA using DNase I (ThermoScientific) and ribosomal RNA with Terminator 5´-Phosphate-dependent

exonuclease (Epicentre), the mRNA-enriched RNA was chemically fragmented to 150~200 bp. Based on these cleaved RNA fragments, cDNA was synthesized using a random hexamer primer and reverse transcriptase. After end reparation and ligation of adaptors, the products were amplified by PCR, further purified, and used to create the final cDNA library. Libraries were sequenced on an Illumina Genome Analyzer IIx. Differential expression analyses were performed through IDEAmex website (http://zazil.ibt.unam.mx/ideamex/) using three methods: edgeR (16), DESeq (17) and NOISeq (18). edgeR and NOISeq were performed by applying TMM (19) as the normalization method. To identify differentially expressed genes, we selected those whose p value was <0.05 and fold change > 2, for each method. Finally, we considered as the best candidates, only genes that appeared differentially expressed in the three methods. The functional annotation of differentially expressed genes regarding the affected pathways was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) (20), using our own R's scripts. RNA-Seq transcriptome data were deposited in the NCBI Gene Expression Omnibus database under accession number GSE113152.

Quantitative real-time RT-PCR (RT-qPCR)

To validate the quality of the sequencing data, some differentially expressed genes were selected for RT-qPCR analysis. mRNA was extracted as described in the section "RNA extraction" and DNA residual was removed using DNase I (Thermo Scientific). cDNA synthesis was performed using RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific). Subsequently, the RT-qPCR was performed using a Maxima SYBR Green/ROXq PCR Master Mix (Thermo Scientific) in a 96-well plate with the Light-Cycler II (Roche). Gene-specific primers used for RT-qPCR are shown in Table 1. *recC* was used

as gene internal standard for PCR amplification. Normalized fold changes of the relative expression ratio were quantified by the $2^{-\Delta\Delta CT}$ method (21). All experiments were performed in triplicates and their average values were calculated.

Cytochrome c content

Membrane fractions of *G. sulfurreducens* were isolated as previously described (22, 23). Outer membrane-enriched fractions were prepared by treating crude membranes with a sarkosyl (sodium N-lauroyl sarcosinate solution at 1% wt/vol) to extract inner membrane proteins. Outer membrane proteins were analyzed by Tris-Glycine denaturing polyacrylamide gel electrophoresis, and *c*-type cytochromes were detected by staining with N,N,N,N-tetramethylbenzidine, as previously described (24, 25). PageRuler pre-stained protein standards were purchased from Thermo Scientific. The Tris-Glycine gel image was digitized using a Gel-doc (Bio Rad).

Immunoblot analysis

Protein extraction from cultures performed under “Pd(II)-reducing” and “Non-Pd(II)-reducing” conditions was conducted by western blot as follows: cells pellets were re-suspended in 150 µl of B-PER II Bacterial Protein extraction reagent (Pierce) and incubated for 15 min. 1.0 mg of total protein per sample was incubated with PAGE-Buffer and boiled for 5 min and were separated on a 15% SDS-PAGE. After separation, proteins were transferred to nitrocellulose membranes (Merck-Millipore) for immunoblot analysis using rabbit polyclonal antibodies raised against *G. sulfurreducens* PilA. Blots were blocked with 3% BSA in PBS overnight at 4°C and then incubated with a 1/1,000 dilution of primary antibody for 4 h at room temperature, washed with PBS, and incubated with a 1/5,000

dilution of goat anti-rabbit alkaline phosphatase-conjugated secondary antibody for 3 h at room temperature. After being washed, blots were developed with 5-bromo-4-chloro-3-indolylphosphatase (BCIP)-Nitro Blue Tetrazolium (Pierce) following the manufacturer's instructions.

Viability assay

Cell viability assays after exposure to Pd(II) was performed by recovering the resting cells in NBAF medium and incubated to measure microbial growth. Prior to inoculation, cell suspensions under Pd(II)-reducing conditions were incubated for 3 h and further transferred from there to NBAF medium with a cellular density of 0.05 (OD 600_{nm}). The cultures were incubated at 30 ^{o}C, and growth was periodically monitored as OD 600_{nm}.

Microbial growth under Pd(II)-reducing conditions

G. sulfurreducens was grown in bicarbonate-buffered medium (26), supplemented with 15 mM pyruvate, 5 mg l^{-1} of Na_2PdCl_4 and 15 mM glutamine. Cultures were incubated at 30 ^{o}C, and Pd(II) reduction, pyruvate consumption and growth were periodically monitored. All experimental treatments were set-up in triplicate.

Analytical techniques

Reduction of Pd(II) was quantified as follows: 5 ml of Pd(II) medium, inoculated or non-inoculated, were filtered using 0.22 μm membrane filters (Millipore, Bedford, USA).

Filtrate samples were then analyzed by inductively coupled plasma-optic emission spectroscopy (ICP-OES, Varian 730-ES).

Pyruvate consumption was measured by a high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series, Santa Clara, CA, USA) equipped with an Aminex HPX-

87H column (BIO-RAD). The column was maintained at 50 °C and was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}. Pyruvate was detected by an UV detector at 210 nm. The standard reagent for quantification was purchased from Sigma-Aldrich (St Louis, MO, USA).

X-ray diffraction analysis

Analysis of the Pd(0) NP's deposited on the different strains of *G. sulfurreducens* was conducted in an X-Ray diffractometer Bruker D8 Advance. Samples were treated as previously described (2). X-ray diffraction (XRD) patterns were recorded from 20°-90° 2θ with a step time of 2 s and step size of 0.01° 2θ.

Results and Discussion

Differentially expressed genes during Pd(II) reduction

Previous studies have shown that *G. sulfurreducens* is able to reduce Pd(II) to Pd(0) NP's (2). However, the proteins involved in this respiratory process have not been identified. To examine the transcriptional changes that occur in *G. sulfurreducens* during Pd(II) reduction, we used RNA-Seq. The p-value and fold change were calculated by the statistical methods DESeq, edgeR and NOISeq. Only genes that showed differential expression by all methods were selected, resulting in 393 differentially expressed genes (Fig. 1A). A cut off p-value <0.05 and a fold change >2 was used.

Out of the 393 genes, 252 displayed statistically significant upregulation (>2 FC), and 141 downregulation under Pd(II)-reducing conditions. This accounted for approximately 11% of the genes in the *G. sulfurreducens* genome, indicating that Pd(II) reduction triggered

significant global gene expression changes. The genes that showed significant differences in transcript levels were classified into the following functional categories: regulatory functions and transcription; energy metabolism and electron transport; DNA metabolism; transport; carbohydrate metabolism; proteolysis; protein synthesis; amino acids metabolism; mobile and extrachromosomal elements; metabolism of cofactors and vitamins; cell envelope; lipid metabolism; unknown function; and others (Fig. 1B).

The most differentially expressed genes were those involved in protein synthesis, where 50 genes were upregulated, while 11 were downregulated. Among the genes upregulated, many are related to tRNA synthesis and ribosomal proteins, such as *rpsU*-1, *rpsB*, *rpsB*, *rpsT*, *rpmB*, *rplU* and *rpsL*. The second group corresponds to genes involved in regulatory functions and transcription with 55 genes (42 upregulated and 13 downregulated). The high number of upregulated transcriptional regulators points out the response of this bacterium to a non-common metal as electron acceptor. The third group of genes is involved in energy metabolism and electron transport (12 upregulated and 29 downregulated), of which 20 code for *c*-type cytochromes.

Expression of *c*-type cytochromes genes during Pd(II) reduction

About half of the differentially expressed genes involved in energy metabolism and electron transport were related to *c*-type cytochromes (9 upregulated and 11 downregulated); 8 are located in the outer membrane, 8 in the periplasmic, 1 in the cytoplasm, and 1 attached to the inner membrane, while the location of the remaining 2 is unknown (Table 2).

227

228 The most upregulated *c*-type cytochromes under Pd(II)-reducing conditions were GSU1062,
229 GSU2808 and PpcA. GSU1062, a putative *c*-type cytochrome, is abundant under ferric
230 citrate reducing conditions (27). Similarly, *gsu2808* encodes for an outer membrane
231 cytochrome that it is reported overexpressed under Fe(III)-reducing conditions and its
232 expression decreases in OmcB deficient conditions (28, 29). On the other hand, cytochrome
233 PpcA participates in electron transfer in the periplasm. A *ppcA* mutant strain showed a
234 decrease in Fe(III) and U(VI) reduction (30, 10). Additionally, the outer membrane
235 cytochromes OmcH and OmcM were also overexpressed under Pd(II)-reducing conditions.
236 Previous work has shown that *omcH* was overexpressed under growing conditions with
237 insoluble Fe(III) oxides; while mutations in the *omcH* and *omcM* genes compromise the
238 reduction of Fe(III) oxides (31). Overexpression of these cytochromes under Pd(II)-
239 reducing conditions suggests that they could be involved in the extracellular reduction of
240 Pd(II).
241 Other *c*-type cytochromes upregulated during Pd(II) reduction were PpcD, GSU2513,
242 GSU2934 and GSU3107. PpcD is overexpressed during the reduction of Mn(IV) oxides,
243 while the *gsu2934* gene is overexpressed during reduction of Fe(III) oxides (31). The
244 putative cytochrome GSU2513 has not previously been reported and its function has yet to
245 be elucidated.
246

247 Surprisingly, during Pd(II) reduction, cytochromes OmcB, OmcC, OmcS and OmcZ, which
248 are involved in the reduction of Fe(III), Mn(IV), and U(VI) (31) were downregulated. A
249 previous model of microbial reduction of Pd(II) by *G. sulfurreducens* suggested that
250 cytochromes OmcB and OmcS, important in the reduction of Fe(III), Mn(IV) or U(VI),

could be involved in the reduction process (8); however, our data suggest that the Pd(II) reduction pathway does not involve those common cytochromes, but others with different biochemical characteristics.

Transcriptional regulation genes expressed in Palladium reduction

The expression profile of some genes encoding proteins involved in transcriptional regulation was considerably altered under Pd(II)-reducing conditions (Table 3). The *gnfK*, *gnfR* and *glnB* genes were upregulated. It has previously been reported that *gnfK*, *gnrR* and *glnB* are upregulated during nitrogen fixation in *G. sulfurreducens* (32). *gnfK* and *gnfR* encode for a un orthodox two-component system, being GnfK a Histidine Kinase and GnrR a Response Regulator. Phosphorylated GnfR, instead of acting as a transcriptional regulator, as the majority of the Response Regulators, binds to the *nifH* mRNA, preventing the formation of a stem-loop structure and therefore avoiding transcription termination (32). In *Geobacter* species, overexpression of genes related to nitrogen fixation has been detected in sediments contaminated with crude oil, groundwater contaminated with uranium, and in microbial fuel cells (33, 34). Under such conditions, where nutrient limitation may exist, it has been proposed that nitrogen fixation is critical for cell growth. Similarly, nitrogen fixation may be an important process during Pd(II) reduction.

Unexpectedly, HgtR was upregulated more than 5.5 times under Pd(II)-reducing conditions. HgtR has been reported as a global regulator for genes involved in biosynthesis and energy generation in *Geobacter* species. Its expression was essential for growth with hydrogen, during which hgtR expression was induced (35). Moreover, it represses the transcription of several genes of the central metabolism and energy generation, such as *gltA* (citrate

274 synthase), *nouA* (NADH dehydrogenase I subunit), *atpG* (ATP synthase FoB '), *srfB*
275 Reductase B' subunit), *icd* (isocitrate dehydrogenase) and *gntR* (35).
276 We observed a decrease in the expression of *gltA*, *icd-mdh*, *atpG* and *nuo* genes, likely by
277 the upregulation of *hgtR* under Pd(II)-reducing conditions. The increase in *hgtR* expression
278 in our experiments was presumably due to the presence of Pd(II), and not to hydrogen,
279 because we used strict anaerobic conditions under a N_2/CO_2 (80/20%) atmosphere without
280 H_2. In addition, *nuoH*-1, *nouD*, *nuoL*-1 and *nuoF*-1, which form part of an operon of 14
281 genes that encode for the NADH dehydrogenase, were also downregulated (see Table S1).
282
283 The transcriptional regulator Fur was also upregulated during Pd(II) reduction. Fur activity
284 is controlled by intracellular levels of Fe(II) and directly regulates the expression of the
285 *feoAB*, *gsu2432*, *gsu2937*, *gsu3274*, *ideR* and *gsu1639* genes (36). However, the regulon
286 Fur also represses genes, such as citrate synthase (*gltA*), isocitrate dehydrogenase (*icd*), 2-
287 oxoglutarate oxidoreductase (*gsu1467*, *gsu1468*, *gsu1469* and *gsu1470*), malate
288 dehydrogenase (*mdh*), NADH (*gsu0346*, *gsu0347*, *gsu0348*, *gsu0349*, *gsu0350*, *gsu0351*)
289 and OmcZ (*gsu2076*) (36). Fur upregulation during Pd(II) reduction may be the result of
290 iron limiting condition, so that cells would have been trying to overexpress genes related to
291 homeostasis and iron uptake. With these results, we can observe that during Pd(II)
292 reduction, *gltA*, *icd*, *mdh*, *gsu1466*, *gsu1468*, *gsu0349* and *omcZ* were downregulated and
293 could be the result of Fur and HgtR overexpression.
294
295 Among the genes coding for possible transcriptional regulators that are upregulated during
296 Pd(II) reduction are *gsu1072*, *gsu0922*, *gsu1639*, *gsu2980*, *gsu3206*, *gsu2185* whose
297 function has not yet been elucidated. Overall, other genes coding for Histidine Kinases and

Response Regulators, as well as transcriptional activators and repressors associated with Pd(II) reduction, were differentially expressed.

Cytochrome *c* and PilA proteins content during palladium reduction

In order to evaluate if mRNA expression correlates with protein content for some *c*-type cytochromes differentially expressed under Pd(II)-reducing conditions, we examined their level by heme-staining of protein gels. As shown in Figure 2A, preparations of soluble fraction, inner membrane and outer membrane proteins, revealed differences in abundance of *c*-type cytochromes in acetate-Pd(II) vs acetate-fumarate conditions. Those bands corresponded to the OmcB and OmcC outer membrane multiheme *c*-type cytochromes, required for Fe(III) reduction in *G. sulfurreducens* (29, 37). OmcS, which is required for Fe(III) oxide reduction (38) and the outer membrane multiheme *c*-type cytochrome, OmcZ, required for optimal current production in microbial fuel cells (39), were abundant in acetate-fumarate in contrast to acetate-Pd(II) conditions.

We also examined the expression of *pilA* gene (GSU1496), which was upregulated under Pd(II)-reducing conditions. To verify the PilA protein content under these conditions, Immunobloting analysis was performed using anti-PilA antibodies. As shown in Figure 2B, PilA was overproduced under these conditions. These results were surprising since the pili is reported to be required for extracellular electron transfer to insoluble electron acceptors, such as metal oxides and electrodes (12), but not to soluble metals.

Contribution of pili on Pd(II) reduction

In *G. sulfurreducens*, the pili is an important structure participating in long-distance extracellular electron transfer to Fe(III) oxides, electron exchange between syntrophic partners, as well as electrodes to generate bioelectricity (12, 40, 41). In order to evaluate the contribution of pili to the reduction of Pd(II) to Pd(0), we studied this process in several mutants that have a direct effect on PilA synthesis. Strain Δ*pilR* does not produce the PilR, which is the major transcriptional activator of the *pilA* gene, which codes for pilin, the structural protein of pili (12); therefore, in this mutant, PilA is severely diminished (23). Thus, Pd(II) reduction in Δ*pilA* and Δ*pilR* mutant strains was quantified. We observed that 98% of Pd(II) is reduced to Pd(0) in the WT strain within the first hour of incubation, while in the Δ*pilA* strain, 81% of Pd(II) was reduced during the same incubation period. When we tested the Δ*pilR* strain, only 61% of Pd(II) reduction was reached after three hours (Fig. 3A). Pd(II) reduction was concomitant with an evident change in color of the biomass, which turned black (Fig. 3A inset). To analyze the nature of the palladium NP's produced, samples of cells covered with these metal were analyzed by XRD (Fig. 3B). The results confirmed the formation of Pd(0) NP's. The pattern of XRD in all samples showed five strong Bragg reflections at 2 < theta > values around 40.11, 46.66, 68.13, 82.11, which correspond to planes (111), (200), (220), and (311) of a face-centered cubic lattice (fcc) (XRD pattern was indexed to ICDD card 89–4897 (fcc palladium syn)). XRD pattern showed that the Pd NPs were crystalline in nature.

341 It has been observed that under U(VI)-reducing conditions, using a Δ*pilA* mutant, the
342 production of some *c*-type cytochrome is diminished, which results in a slight decrease in
343 the reduction of U(VI) to U(IV) (42). It has been suggested that the slight reduction of
344 U(VI) in the pili-deficient strain is due to the decrease in outer membrane *c*-type
345 cytochromes and not to pili deficiency (43). Therefore, the slight negative effect on the
346 reduction of Pd(II) by the Δ*pilA* strain, in our experiments, could be due to a decrease in the
347 production of outer membrane *c*-type cytochromes, rather than a negative effect on the pili
348 itself, similarly to reports on U(VI) reduction (43). Since PilR is a transcriptional regulator
349 that controls the expression of at least 44 genes, among which are several *c*-type
350 cytochromes, we propose that the decreased in Pd(II) reduction observed in the Δ*pilR*
351 mutant strain could be related to *c*-type cytochrome content rather than to pili absence.
352 Pd(II) is a toxic metal for many microorganisms; it may inhibit the activity of creatine
353 kinase, aldolase, succinate dehydrogenase, carbonic anhydrase, alkaline phosphatase and
354 prolyl hydroxylase (44). Pd(II) has been found to be toxic in *Shewanella oneidensis*;
355 however, exposed cells could be recovered when a suitable electron donor was provided (1).
356 Similar to *S. oneidensis*, *G. sulfurreducens* could recover viability after the reduction of
357 Pd(II) in NBAF medium, as shown in supplementary data (Fig. S1). In addition, the Δ*pilA*
358 and Δ*pilR* mutant strains took slightly more time than the WT strain to recover viability
359 after exposure to Pd(II) (Fig. S1), which may be due to increased exposure to Pd(II),
360 resulting from the decreased capacity of these strains to reduce this metal (Fig. 3A).
361

Growth of *Geobacter sulfurreducens* coupled to Pd(II) reduction

Because the biological reduction of Pd(II) by *S. oneidensis*, *D. desulfuricans* and *G. sulfurreducens* was carried out with resting cells, it is unknown whether this process can be coupled to microbial growth (3, 1, 2). In order to evaluate if *G. sulfurreducens* can couple growth to Pd(II) reduction, incubations were performed in FW medium (see experimental procedures) using acetate (20 mM), lactate (15 mM) or pyruvate (15 mM) as electron donors, and Pd(II) (5 mg/mL) as electron acceptor. No microbial growth was observed with acetate and lactate, which may be due to the fact that the RNA-Seq results showed that the *gltA* (citrate synthase) and *mdh* (malate dehydrogenase) genes were downregulated during Pd(II) reduction, possibly exerting a negative effect on the TCA cycle and therefore on the production of energy. However, by providing pyruvate as an electron donor, we would expect that *G. sulfurreducens* could use an alternative pathway to that of acetate metabolism, redirecting it to biomass biosynthesis (45). However, under these conditions, neither growth nor Pd(II) reduction was observed (data not shown). To verify if the lack of microbial growth observed, could be due to a limitation in the nitrogen source, because it is worth noting that, under Pd(II)-reducing conditions, we observed the overexpression of genes related to nitrogen fixation (*gnfK*, *gnfR* and *glnB*), glutamine was also added to the culture medium. Interestingly, under these conditions, we observed microbial growth in pyruvate-Pd(II) medium (Fig. 4A). Moreover, this growth was coupled to the reduction of 90% Pd(II) to Pd(0) (Fig. 4B) and to the consumption of up to 3 mM of pyruvate (Fig. 4C). Our results imply complex linkages between central metabolism and Pd(II) reduction in *G. sulfurreducens.* Future research is necessary to identify which nutritional elements are conditioning factors to favor the bacterial growth in the presence of other rare metals.

385 However, to our knowledge, this paper reports, for the first time, the microbial reduction of
386 Pd(II) coupled to growth.

387 Validation of selected differentially expressed genes using RT-qPCR

388 To verify results obtained from RNA-Seq experiments and to get quantitative data to
389 compare the transcript abundances under Pd(II)-reducing conditions, we performed RT-
390 qPCR analyses from 24 selected genes encoding proteins involved in electron transfer,
391 transcriptional regulators and central metabolism (Table 4**)**. These genes included the *c*-
392 type cytochromes genes (*omcH*, *omcM*, *omcB*, *omcC*, *omcS*, *omcZ*, *ppcD*, *gsu1062*, *ppcB*,
393 *omcE*, *ccpA*, *gsu0615*, *gsu2937*, *gsu2513*, *gsu2808*, *omcQ* and *gsu2495*) and the cold shock
394 DNA/RNA-binding protein, *gsu0207*, the transcriptional regulators, *hgtR* and *gsu0837*, the
395 menaquinol oxidoreductase complex Cbc3, *gsu1650*, the pilin protein, *pilA*, the NADH
396 dehydrogenase I, *nuoH*-1, and the citrate synthase I, *gltA*.
397
398 Upregulation of *omcH*, *omcM*, *gsu1062*, *gsu2513*, *gsu2808* and *ppcD* genes that encoded
399 for *c*-type cytochromes under Pd(II)-reducing conditions was confirmed by RT-qPCR.
400 Similarly, the expression of *pilA*, *gsu0207*, *hgtR* y *gsu1650* was high under these conditions
401 according to the RT-qPCR. On the other hand, the low transcription of *omcB*, *omcC*, *omcS*,
402 *omcZ*, *gsu0837*, *gsu0345*, *gsu2813*, *gsu0615*, *gsu2937*, *omcQ*, *gsu2495* and *gsu1106*
403 observed in RNA-Seq analyses was confirmed by RT-qPCR. Furthermore, the low
404 transcription of *nuoH*-1 and *gltA* genes was also observed, in agreement with the high
405 expression of *hgtR*, a negative regulator for these genes (35).
406

407 Model for biological Pd(II) reduction by *Geobacter sulfurreducens*

Biological reduction of Pd(II) is a poorly studied process. To date, several bacteria have been reported with the ability to reduce Pd(II) to Pd(0) NP's (46). In *Desulfovibrio fructosivorans* and *Escherichia coli*, the biological reduction of Pd(II) to Pd(0) is linked to the activity of a hydrogenase (47, 48). *Shewanella oneidensis* and *D. desulfuricans* present a similar Pd(II) reduction mechanism, suggesting that hydrogenase and cytochrome c3 are involved in the reduction process (3, 46).

Based on the results shown here, we propose the following Pd(II) reduction model: Electrons derived from the anaerobic metabolism are transferred through the electron carriers NADH dehydrogenase and MQ to the periplasmic cytochrome MacA, which transfers the electrons to the tri-heme-periplasmic cytochromes PpcA/PpcD. PpcA/PpcD then transport the electrons to the outer membrane cytochromes GSU2513, GSU2808, OmcM and OmcH, which ultimately reduce Pd(II) to Pd(0) (Fig. 5). In addition, the periplasmic cytochromes GSU1062 and CbcN could be intermediates in electron transfer from MacA and/or PpcA/PpcD to the outer membrane cytochromes. Future analyses are necessary to elucidate whether Pd(II) reduction proceeds in the periplasm.

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References

1. De Windt W, Boon N, Van den Bulcke J, Rubberecht L, Prata F, Mast J, Hennebel T, Verstraete W. 2006. Biological control of the size and reactivity of catalytic Pd(0) produced by *Shewanella oneidensis*. *Antonie van Leeuwenhoek* 90:377-389.

2. Pat-Espadas AM, Razo-Flores E, Rangel-Mendez JR, Cervantes FJ. 2013. Reduction of palladium and production of nano-catalyst by *Geobacter sulfurreducens*. *Appl Microbiol Biotech* 97:9553–9560.

3. Lloyd JR, Yong P, Macaskie LE. 1998. Enzymatic recovery of elemental palladium by using sulfate-reducing bacteria. *Appl Environ Microbiol* 64(11):4607–4609.

4. Caccavo F, Lonergan DJ, Lovley DR, Davis M, Stolz JF, McInerney MJ. 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen-and acetate-oxidizing dissimilatory metal-reducing microorganism. *Appl Environ Microbiol* 60:3752–3759.

5. Sanford RA, Wu Q, Sung Y, Thomas SH, Amos BK, Prince EK, Löffler FE. 2007. Hexavalent uranium supports growth of *Anaeromyxobacter dehalogenans* and *Geobacter* spp. with lower than predicted biomass yields. *Environ Microbiol* 9(11):2885–2893.

452 6. Law N, Ansari S, Livens FR, Renshaw JC, Lloyd JR. 2008. Formation of nanoscale
453 elemental silver particles via enzymatic reduction by *Geobacter sulfurreducens*. *Appl*
454 *Environ Microbiol* 74(22):7090–7093.
455
456 7. Lovley DR, Ueki T, Zhang T, Malvankar NS, Shrestha PM, Flanagan KA, Aklujkar M,
457 Butler JE, Giloteaux L, Rotaru AE, Holmes DE, Franks AE, Orellana R, Risso C, Nevin K
458 P. 2011. Geobacter: the microbe electric's physiology, ecology, and practical applications.
459 *Adv Microb Physiol* 59:1-100.
460
461 8. Pat-Espadas AM, Razo-Flores E, Rangel-Mendez JR, Cervantes FJ. 2014. Direct and
462 Quinone-Mediated Palladium Reduction by *Geobacter sulfurreducens*: Mechanisms and
463 Modeling. *Environ Sci Technol* 48(5):2910–2919.
464
465 9. Ding YH, Hixson KK, Aklujkar MA, Lipton MS, Smith RD, Lovley DR, Mester T. 2008.
466 Proteome of *Geobacter sulfurreducens* grown with Fe(III) oxide or Fe(III) citrate as the
467 electron acceptor. *Biochim Biophys Acta* 1784(12): 1935–1941.
468
469 10. Mehta T, Coppi MV, Childers SE, Lovley DR. 2005. Outer membrane *c*-type
470 cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*.
471 *Appl Environ Microbiol* 71(12):8634–8641.
472
473 11. Childers SE, Ciufo S, Lovley DR. 2002. *Geobacter metallireducens* accesses insoluble
474 Fe(III) oxide by chemotaxis. *Nature* 416 (6882):767–769.

475 12. Reguera G, McCarthy KD, Mehta T, Nicoll JS, Tuominen MT, Lovley DR. 2005.
476 Extracellular electron transfer via microbial nanowires. *Nature* 435:1098-1101.
477
478 13. Shelobolina ES, Coppi MV, Korenevsky AA, DiDonato LN, Sullivan SA, Konishi H,
479 Xu H, Leang C, Butler JE, Kim BC, Lovley DR. 2007. Importance of *c*-type cytochromes
480 for U(VI) reduction by *Geobacter sulfurreducens*. *BMC Microbiol* 7:16.
481
482 14. Shi L, Squier TC, Zachara JM, Fredrickson JK. 2007. Respiration of metal (hydr)oxides
483 by *Shewanella* and *Geobacter* : a key role for multihaem c -type cytochromes. *Mol*
484 *Microbiol* 65(1):12–20.
485
486 15. Coppi MV, Leang C, Sandler SJ, Lovley DR. 2001. Development of a genetic system
487 for *Geobacter sulfurreducens*. *Appl Environ Microbiol* 67(7):3180-3187.
488
489 16. Robinson MD, McCarthy DJ, Smyth GK. 2010. EDGER: a Bioconductor package for
490 differential expression analysis of digital gene expression data. *Bioinformatics* 26:139-140.
491
492 17. Anders S, Huber W. 2010. Differential expression analysis for sequence count data.
493 *Genome Biol* 11(10):R106.
494
495 18. Tarzona S, García-Alcalde F, Dopazo J, Ferrer A, Conesa A. 2011. Differential
496 expression in RNA-Seq: a matter of depth. *Genome Res* 21(12): 2213-2223.
497

498 19. Robinson MD, Oshlack A. 2010. A scaling normalization method for differential
499 expression analysis of RNA-Seq data. *Genome Biol* 11(3): R25.
500
501 20. Kanehisa M, Goto S. 2000. KEGG: Kyoto Encyclopedia of genes and genomes.
502 *Nucleic Acids Res* 28(1):27-30.
503
504 21. Livak K, Shmittgen TD. 2001. Analysis of relative gene expression data using real-time
505 quantitative PCR and the 2-(Delta Delta C(T)) method. *Methods* 25:402–408.
506
507 22. Kim BC, Leang C, Ding YH, Glaven RH, Coppi MV, Lovley DR. 2005. OmcF, a
508 putative *c*-type monoheme outer membrane cytochrome required for the expression of other
509 outer membrane cytochromes in *Geobacter sulfurreducens*. *J Bacteriol* 187(13):4505-4513.
510
511 23. Juárez K, Kim BC, Nevin K, Olvera L, Reguera G, Lovley DR, Methé BA. 2009. PilR,
512 a transcriptional regulator for pilin and other genes required for Fe(III) reduction in
513 *Geobacter sulfurreducens*. *J Mol Microbiol Biotechnol* 16:146-158.
514
515 24. Thomas PE, Ryan D, Levin W. 1976. An improved staining procedures for the
516 detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate
517 polyacrylamide gels. *Anal Biochem* 75:168-176.
518
519 25. Francis RT Jr, Becker RR. 1984. Specific indication of hemoproteins in polyacrylamide
520 gel using a double-staining process. *Anal Biochem* 136(2):509-514.

521 26. Lovley DR, Giovannoni SJ, White DC, Champine JE, Phillips EJP, Gorby YA,
522 Goodwin S. 1993. *Geobacter metallired*ucens gen. nov. sp. nov., a microorganism capable
523 of coupling the complete oxidation of organic compounds to the reduction of iron and other
524 metals. *Arch Microbiol* 159(4):336–344.
525
526 27. Ding YH, Hixson KK, Giometti CS, Stanley A, Esteve-Nuñez A, Khare T, Tollaksen
527 SL, Zhu W, Adkins JN, Lipton MS, Smith RD, Mester T, Lovley DR. 2006. The proteome
528 of dissimilatory metal-reducing microorganism *Geobacter sulfurreducen*s under various
529 growth conditions. *Biochim Biophys Acta* 1764(7):1198-1206.
530
531 28. Methé BA, Webster J, Nevin K, Butler J, Lovley DR. 2005. DNA Microarray analysis
532 of nitrogen fixation and Fe(III) reduction in *Geobacter sulfurreducens*. *App Environ*
533 *Microbiol* 71(5): 2530-2538.
534
535 29. Leang C, Adams LA, Chin KJ, Nevin KP, Methé BA, Webster J, Sharma ML, Lovley
536 DR. 2005. Adaptation to disruption of the electron transfer pathway for Fe(III) reduction in
537 *Geobacter sulfurreducens*. *J Bacteriol* 187(17):5918-5926.
538
539 30. Lloyd JR, Leang C, Hodges MAL, Coppi MV, Cuifo S, Methe B, Sandler SJ, Lovley
540 DR. 2003. Biochemical and genetic characterization of PpcA, a periplasmic *c*-type
541 cytochrome in *Geobacter sulfurreducens*. *Biochem J* 369(1):153-161.
542
543 31. Aklujkar M, Coppi MV, Leang C, Kim BC, Chavan MA, Perpetua LA, Giloteaux L,
544 Liu A, Holmes DE. 2013. Proteins involved in electron transfer to Fe(III) and Mn(IV)

545 oxides by *Geobacter sulfurreducens* and *Geobacter uraniireducens*. *Microbiol* 159:515-
546 535.
547
548 32. Ueki T, Lovley DR. 2010. Novel regulatory cascades controlling expression of
549 nitrogen-fixation genes in *Geobacter sulfurreducens*. *Nucleic Acids Res* 38(21):7485-7499.
550
551 33. Holmes DE, Nevin KP, Lovley DR. 2004. In situ expression of *nifD* in Geobacteraceae
552 in subsurface sediments. *Appl Environ Microbiol* 70:7251-7259.
553
554 34. Holmes DE, O´Neil RA, Chavan MA, N´Guessan LA, Vrionis HA, Perpetua LA,
555 Larrahondo MJ, DiDonato R, Liu A, Lovley DR. 2009. Transcriptome of *Geobacter*
556 *uraniireducens* growing in uranium-contaminated subsurface sediments. *ISME J* 3:216-230.
557
558 35. Ueki T, Lovley DR. 2010. Genome-wide gene regulation of biosynthesis and energy
559 generation by a novel transcriptional repressor in Geobacter species. *Nucleic Acids Res*
560 38(3):810-821.
561
562 36. Embree M, Qiu Y, Shieu W, Nagajaran H, O´Neil R, Lovley DR, Zengler K. 2014. The
563 iron stimulon and Fur regulon of *Geobacter sulfurreducens* and their role in energy
564 metabolism. *App Environ Microbiol* 80(9):2918-2927.
565
566 37. Liu Y, Fredrickson JK, Zachara JM, Shi L. 2015. Direct involvement of ombB, omaB,
567 and omcB genes in extracellular reduction of Fe(III) by *Geobacter sulfurreducens* PCA.
568 *Front Microbiol* 6:1075.

569

570 38. Qian X, Mester T, Morgado L, Arakawa T, Sharma ML, Inoue K, Joseph C, Salgueiro
571 CA, Maroney MJ, Lovley DR. 2011. Biochemical characterization of purified OmcS, a *c*-
572 type cytochrome required for insoluble Fe(III) reduction in *Geobacter sulfurreducens*.
573 *Biochim Biophys Acta* 1807(4):404-412.

574

575 39. Inoue K, Leang C, Franks AE, Woodard TL, Nevin KP, Lovley DR. 2011. Specific
576 localization of the *c*-type cytochrome OmcZ at the anode surface in current-producing
577 biofilms of *Geobacter sulfurreducens*. *Environ Microbiol Rep* 3(2):211-217.

578

579 40. Smith JA, Lovley DR, Tremblay PL. 2013. Outer cell surface components essential for
580 Fe(III) oxide reduction by *Geobacter metallireducens*. *Appl Environ Microbiol* 79:901–907.

581

582 41. Summers ZM, Fogarty HE, Leang C, Franks AE, Malvankar NS, Lovley DR. 2010.
583 Direct exchange of electrons within aggregates of an evolved syntrophic coculture of
584 anaerobic bacteria. *Science* 330:1413–1415.

585

586 42. Cologgi DL, Lampa-Pastirk S, Speers AM, Kelly SD, Reguera G. 2011. Extracellular
587 reduction of uranium via *Geobacter* conductive pili as a protective cellular mechanism.
588 *Proc Natl Acad Sci* 108(37):15248 –15252.

589

590 43. Orellana R, Leavitt J, Comolli LR, Csencsits R, Janot N, Flanagan KA, Gray AS, Leang
591 C, Izallalen M, Mester T, Lovley DR. 2013. U(VI) Reduction by diverse outer surface *c*-
592 type cytochromes of *Geobacter sulfurreducens*. *Appl Environ Microbiol* 79:6369–6374.

593

594 44. Liu TZ, Lee SD, Bhatnagar RS. 1979. Toxicity of palladium. *Toxicol Lett* 4(6):469–473.

595

596 45. Wilkins MJ, Wrighton KC, Nicora CD, Williams KH, McCue LA, Handley KM, Miller
597 C S, Giloteaux L, Montgomery AP, Lovley DR, Banfield JF, Long PE, Lipton MS. 2013.
598 Fluctuations in species-level protein expression occur during element and nutrient cycling
599 in the subsurface. *PLoS One* 8(3): e57819

600

601 46. De Corte S, Hennebel T, De Gusseme B, Verstraete W, Boon N. 2012. Bio-palladium:
602 from metal recovery to catalytic applications. *Microb Biotechnol* 5(1):5-17.

603

604 47. Mikheenko IP, Rousset M, Dementin S, Macaskie LE. 2008. Bioaccumulation of
605 palladium by *Desulfovibrio fructosivorans* wild-type and hydrogenase-deficient strains.
606 *Appl Environ Microbiol* 74: 6144–6146.

607

608 48. Deplanche K, Caldelari I, Mikheenko IP, Sargent F, Macaskie LE. 2010. Involvement
609 of hydrogenases in the formation of highly catalytic Pd(0) nanoparticles by bioreduction of
610 Pd(II) using *Escherichia coli* mutant strains. *Microbiol* 156(9):2630-2640.

611

612 49. Gardy JL, Spencer C, Wang K, Ester M, Tusnády GE, Simon I, Hua S, deFays K,
613 Lambert C, Nakai K, Brinkman FS. 2003. PSORT-B: Improving protein subcellular
614 localization prediction for Gram-negative bacteria. *Nucleic Acids Res* 31(13):3613-3617.

615

616 50. Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell
617 using TargetP, SignalP, and related tools. *Nat Protoc* 2(4):953-971.

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619 **Figure Legends**

620 **FIG 1.** Transcriptome analysis results from *Geobacter sulfurreducens* under Pd(II)-
621 reducing conditions. (A) Venn diagram representing differential gene expression analysis
622 from Pd(II)-reducing conditions by three statistical methods. (B) Functional overview of
623 the genes that were differentially expressed during Palladium reduction.

624

625 **FIG 2.** *c*-type cytochrome and PilA protein content found under Pd(II)-reducing conditions.
626 (A) SDS-PAGE heme stained. Outer membrane (OM), inner membrane (IM) and soluble
627 fraction (SF) were prepared from PCA strain with acetate-fumarate (Ac-F) or acetate-Pd(II)
628 (Ac-Pd(II)). The localization of OmcC, OmcB, OmcS, OmcZ and PpcA were labeled based
629 on expected molecular weight (78.96, 74.89, 42.94, 47.09 and 7.72 kDa, respectively). (B)
630 Immunoblot analysis for PilA. The PageRuler Pre-stained Protein Ladder standard
631 (ThermoScientific) was used as a molecular weight.

632

633 **FIG 3.** Palladium reduction by different strains of *G. sulfurreducens*. (A) Kinetics of Pd(II)
634 reduction. Photograph of bottles applied for Pd(II) reduction is shown in the inset. (B)
635 Comparison of XRD patters corresponding to cells and black precipitates obtained from
636 cultures of WT, Δ*pilA* and Δ*pilR* strains under Pd(II)-reducing conditions. In (A) and (B),
637 WT strain line blue, Δ*pilA* strain line black, Δ*pilR* strain line orange.

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FIG 4. Growth of *Geobacter sulfurreducens* coupled to Pd(II) reduction. (A) Growth. (B) Pd(II) reduction. (C) Consumption of pyruvate. In A, B and C, line with squares, pyruvate-Pd(II) supplemented with glutamine; line with rhombi, pyruvate-Pd(II).

FIG 5. Model of Pd(II) reduction by *Geobacter sulfurreducens*.

TABLE 1 Bacterial strain and primer sequences used in this work and for RT-qPCR validations

Strain	Description	Reference/source
Geobacter sulfurreducens		
PCA strain	Wild type	4
Δ*pilR* strain	*pilR*::kan, kanamicin resistance	23
Δ*pilA* strain	*pilA*::kan, kanamicin resistance	12
Oligonucleotides	**Sequence 5´➔3´**	
qPCRrecCfw	CTGTCGTCACCCTTTGTTCC	This study
qPCRrecCrev	GAAAGGGATAGGAGCCGTTC	This study
qPCRomcMfw	TGGAGACTACCCATGCTGAA	This study
qPCRomcMrev	AGACGTCGAGGTGCTCGTAT	This study
qPCRomcHfw	ATGGACGTGAATGGAAGGAG	This study
qPCRomcHrev	TGGCAGTCAGTACAGGTGGA	This study

qPCRppcDfw	CAGCACTCACCCTGTTCTGT	This study
qPCRppcDrev	TGCTTTTTGTGGTCGAAGGT	This study
qPCRhgtRfw	GAGAGAAAATCCGCGGTACA	This study
qPCRhgtRrev	TAGCCTCCCTCATGATGTCC	This study
qPCRGSU1062fw	TCCAGGATTCCGAAACTCAA	This study
qPCRGSU1062rev	CGGCTCTATTTCGCTCTCAG	This study
qPCRGSU0207fw	AGCAAGGGCTTTGGTTTTCT	This study
qPCRGSU0207rev	TTACCCTATCGCCTTCAGCA	This study
qPCRGSU1650fw	TACCCCGTGTTTCGCTATCT	This study
qPCRGSU1650rev	CCGTGGAAATCGAAGAATTT	This study
qPCRGSU0837fw	GCATTGAACGGATCTTCGAC	This study
qPCRGSU0837rev	GCGGACGTACTGTTCATGTC	This study
qPCRGSU0345fw	CGAGTCACTCTCGTTGCAGA	This study
qPCRGSU0345rev	TGCGAGAGAGCAGATGAAGA	This study
qPCRGSU2813fw	CGGTTCTCAACTCGGTCTTC	This study
qPCRGSU2813rev	GACATAGTCGGGGATGCTGT	This study
qPCRGSU0615fw	ACTTTGCCCTCTGTTTCACG	This study
qPCRGSU0615rev	GAATCCGCTCTTTGACAAGC	This study
qPCRGSU2937fw	GTCACCAGGAAGTCCACGAT	This study
qPCRGSU2937rev	TCAGGTGCTCAACTTTCACG	This study
qPCRGSU1106fw	AGTTCCCCAGTACGTGTTCG	This study
qPCRGSU1106rev	GCGAACTTGGAATCCTTTTG	This study
qPCRomcBfw	GGAGTATGTGGCATCCCTTG	This study

qPCRomcBrev	ACCGTTGGCATTCGTATCTC	This study
qPCRomcCfw	AGAGTACGTGGCATCCCTTG	This study
qPCRomcCrev	CCGTTGGCATTCGTATCTCT	This study
qPCRomcSfw	TCCTACCAGAACAGCAACGA	This study
qPCRomcSrev	ATAGGAACCGCTCAGGGACT	This study
qPCRomcZfw	AAGCCGACTGTCTCGAGTGT	This study
qPCRomcZrev	CGGAGGTATTGATGCAGCTT	This study
qPCRomcEfw	CCAGATCTGCGTGTTCTGTC	This study
qPCRomcErev	CATGCTGCTGGACGAGTAGA	This study
qPCRppcBfw	CCACAAGAAACACCAGACGA	This study
qPCRppcBrev	CACCCCTTGCAGGACTTG	This study
qPCRpilAfw	AATTACCCCCATACCCCAAC	This study
qPCRpilArev	AGCAGCTCGATAAGGGTGAA	This study
qPCRGSU2513Fw	GACCAGGCCCAGTTCAAGTA	This study
qPCRGSU2513Rv	GGTGTCGATTTCAATTTCCTG	This study
qPCRGSU2808Fw	CGAAATGCCATACCTCCACT	This study
qPCRGSU2808Rv	TATATTGCCCCGAGTTGTCC	This study
qPCRGSU0592Fw	GGAGTATTCCCCCTGAAGGA	This study
qPCRGSU0592Rv	ATGTTTCTGGTGGCTGAAGG	This study
qPCRGSU2495Fw	ACCAGCTCTGCTTCGACTGT	This study
qPCRGSU2495Rv	GGTTGTGGCAGAGGGTACAT	This study

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659 **TABLE 2.** *c*-type cytochrome and putative cytochrome genes that were differentially expressed in palladium reduction

Cytochromes genes that were upregulated in RNA-Seq experiments

Locus ID	Gene annotation	Gene name	Fold change	log2 FC	P-*value*	Signal peptide	Cellular location
GSU0612	Cytochromec3, 3 heme-binding sites	*ppcA*	0.381	1.390	1.4E-06	Yes	Periplasmic
GSU1024	Cytochromec3, 3 heme-binding sites	*ppcD*	0.440	1.186	2.4E-03	Yes	Periplasmic
GSU1062	Cytochrome c putative, 1 heme-binding site	-	0.281	1.830	4.1E-05	Yes	Periplasmic
GSU2513	Cytochrome c family protein, 1 heme-binding site	-	0.423	1.243	2.4E-02	Yes	Unknown
GSU2808	Cytochrome c family protein, 5 heme-binding sites	-	0.338	1.563	3.0E-05	Yes	Outer membrane
GSU2934	Cytochrome c family protein, 9 heme-binding sites	*cbcN*	0.476	1.071	2.6E-04	Yes	Periplasmic
GSU3107	Ribosomal protein L31, 1 heme-binding site	*rpmE*	0.404	1.307	5.5E-03	NO	Cytoplasmic
GSU2883	Cytochrome c family protein, 18 heme-binding sites	*omcH*[a]	0.789	0.342	1.0E+00	Yes	Outer membrane
GSU2294	Cytochrome c family protein, 4 heme-binding sites	*omcM*[a]	0.530	0.917	5.8E-02	Yes	Outer membrane

Cytochromes genes that were downregulated in RNA-Seq experiments

Locus ID	Gene annotation	Gene name	Fold change	log2 FC	P-*value*	Signal peptide	Cellular location
GSU0283	Sensor histidine kinase, 1 heme-binding site	-	2.442	-1.288	7.9E-05	NO	Cytoplasmic membrane
GSU0592	Cytochrome c family protein, 11 heme-binding sites	*omcQ*	2.491	-1.317	1.6E-05	Yes	Outer membrane
GSU0615	Cytochrome c family protein, 5 heme-binding sites	-	4.283	-2.099	1.9E-03	Yes	Periplasmic
GSU2076	Cytochrome c family protein, 5 heme-binding sites	*omcZ*	2.181	-1.125	4.8E-06	Yes	Outer membrane
GSU2495	Cytochrome c family protein, 22 heme-binding sites	-	3.333	-1.737	2.5E-03	NO	Unknown
GSU2731	Polyheme membrane-associated cytochrome c, 8 heme-binding sites	*omcC*	2.125	-1.088	2.3E-03	Yes	Outer membrane
GSU2737	Polyheme membrane-associated cytochrome c, 8 heme-binding sites	*omcB*	4.047	-2.017	4.6E-02	Yes	Outer membrane
GSU2811	Cytochrome c Hsc, 1 heme-binding site	*cccA*	2.657	-1.410	6.8E-16	Yes	Periplasmic
GSU2813	Cytochrome c551 peroxidase, 2 heme-binding sites	*ccpA*	3.285	-1.716	4.5E-13	Yes	Periplasmic
GSU2937	Cytochrome c family protein, 5 heme-binding sites	-	3.523	-1.817	9.9E-03	Yes	Periplasmic
GSU2504	Cytochrome c family protein, 1 heme-binding site	*omcS*[a]	1.829	-0.871	1.0E+00	Yes	Outer membrane

660 Protein cellular location was predicted with PSORT-B (49) and SignalP 3.0 Server (50).

661 [a] values are out of cut off for Fold change but validated by RT-qPCR

662

663 **TABLE 3.** Genes encoding transcriptional regulators differentially expressed in during palladium reduction

Genes encoding proteins involved in transcriptional regulation that were upregulated in RNA-Seq experiments

Locus ID	Gene annotation	Gene name	Fold change	log FC	P-*value*
GSU0284	TraR/DksA family, zinc finger transcriptional regulator	*dksA*	0.324	1.63	5.7E-05
GSU0298	Two-component system	*fgrM-N*	0.347	1.53	8.0E-23
GSU0475	Sensor histidine kinase, PAS domain-containing	-	0.401	1.32	1.4E-02
GSU0596	Response receiver	-	0.304	1.72	2.1E-11
GSU0849	Regulator of cell morphogenesis and NO signaling	*scdA*	0.328	1.61	2.8E-03
GSU0863	Regulatory protein	-	0.292	1.78	2.7E-03
GSU0877	Response regulator, PilZ domain-containing	-	0.203	2.30	7.4E-04
GSU0941	Nitrogen fixation transcript antitermination sensor histidine kinase	*gnfK*	0.134	2.90	6.1E-08
GSU0962	Two-component system, NtrC family, sensor kinase	-	0.186	2.42	1.5E-04
GSU1038	Response receiver histidine kinase	-	0.338	1.57	6.2E-03
GSU1072	IclR family, transcriptional regulator	-	0.070	3.83	2.3E-08
GSU1117	Response regulator	-	0.243	2.04	3.5E-04

GSU1345	BadM/Rrf2 family, transcriptional regulator	-	0.203	2.30	2.5E-03
GSU1379	Transcriptional regulator, ferric uptake regulator	*fur*	0.275	1.86	1.0E-07
GSU1419	Cro/CI family, transcriptional regulator	-	0.278	1.85	5.3E-04
GSU1521	Integration host factor, alpha subunit	*ihfA*-1	0.250	2.00	2.8E-14
GSU1639	BadM/Rrf2 family, transcriptional regulator	-	0.082	3.61	1.6E-16
GSU1658	Response regulator receiver modulated diguanylate cyclase	-	0.263	1.93	1.9E-07
GSU1836	Nitrogen regulatory protein P-II	*glnB*	0.094	3.41	1.0E-16
GSU1999	RNA-binding protein Hfq	*hfq*	0.481	1.06	2.5E-03
GSU2016	Sensor diguanylate cyclase/phosphodiesterase	-	0.428	1.23	5.7E-03
GSU2046	Response regulator	-	0.436	1.20	5.0E-05
GSU2287	response regulator	-	0.358	1.48	4.6E-05
GSU2288	Sensor histidine kinase	-	0.298	1.75	1.4E-03
GSU2384	sensor histidine kinase, GAF domain-containing	-	0.275	1.86	2.3E-03
GSU2625	ArsR family, transcriptional regulator	-	0.194	2.36	2.0E-03
GSU2641	PATAN domain GTPase-activating protein	-	0.138	2.86	1.4E-33
GSU2666	TetR family, transcriptional regulator	-	0.443	1.17	1.3E-05

GSU2735	TetR family, transcriptional regulator	-	0.268	1.90	1.8E-03
GSU2741	TetR family, transcriptional regulator	-	0.200	2.32	1.8E-02
GSU2809	Fur family, transcriptional regulator	-	0.333	1.59	1.3E-05
GSU2822	Response regulator receiver and ANTAR domain protein	*gnfR*	0.055	4.19	4.5E-06
GSU2980	CopG family transcriptional regulator, nickel-responsive regulator	*nikR*	0.117	3.09	2.9E-04
GSU3127	AraC family, transcriptional regulator	-	0.441	1.18	5.0E-02
GSU3206	TraR/DksA family, zinc finger transcriptional regulator	-	0.147	2.77	9.0E-07
GSU3261	Response regulator	-	0.439	1.19	2.9E-04
GSU3292	Fur family, transcriptional regulator	-	0.438	1.19	1.9E-07
GSU3298	Cro/CI family, transcriptional regulator	-	0.208	2.26	8.4E-04
GSU3364	Transcriptional regulator	*hgtR*	0.021	5.54	3.6E-16
GSU2185	FlgM family, anti-sigma-28 factor	-	0.135	2.89	9.9E-07

Genes encoding proteins involved in transcriptional regulation that were downregulated in RNAseq experiments

Locus ID	Gene annotation	Gene name	Fold change	log FC	P-*value*
GSU0013	MarR family, transcriptional regulator	-	3.228	-1.69	5.9E-08
GSU0452	Sensor histidine kinase	-	2.315	-1.21	4.5E-04
GSU0534	BadM/Rrf2 family, transcriptional regulator	*iscR-1*	2.065	-1.05	5.8E-12
GSU0537	Sensor diguanylate cyclase/phosphodiesterase, PAS domain-containing	-	2.134	-1.09	1.1E-03
GSU0811	Fis family, transcriptional regulator	-	3.363	-1.75	4.1E-02
GSU0836	RNA polymerase-binding protein Rnk	*rnk-2*	91.515	-6.52	4.2E-28
GSU0837	Response regulator	-	99.620	-6.64	2.0E-30
GSU0841	NtrC family, response regulator	-	39.998	-5.32	4.3E-04
GSU0842	Sensor histidine kinase response regulator, GAF and PAS domain-containing	-	15.129	-3.92	3.6E-46
GSU1119	Response receiver histidine kinase	-	2.417	-1.27	4.7E-03
GSU1671	Response receiver-modulated diguanylate cyclase	-	2.223	-1.15	5.6E-04
GSU2602	Integration host factor, subunit beta	*ihfB-2*	3.979	-1.99	1.1E-02
GSU2815	Sensor histidine kinase, PAS domain-containing	-	2.419	-1.27	3.3E-02

664 **TABLE 4.** Expression of genes with relevant phenotype observed in RNA-Seq analysis

665 validated by RT-qPCR

Locus ID	Common name	Avg +Pd/Avg -Pd
GSU2294	OmcH, cytochrome c	2.87
GSU2883	OmcM, cytochrome c	1.29
GSU1024	PpcD, cytochrome c	5.80
GSU1062	Cytochrome c, 1 heme-binding site	5.63
GSU0207	Cold shock DNA/RNA-binding protein	12.91
GSU1650	Cytochrome b/b6 complex	2.14
GSU3364	HgtR, hydrogen-dependent growth transcriptional regulator	9.42
GSU1945	PilA, pilin protein	1.50
GSU2513	Cytochrome c, 1 heme-binding site	1.91
GSU2808	Cytochrome c, 5 heme-binding site	9.44
GSU2737	OmcB, lipoprotein cytochrome c	0.28
GSU3731	OmcC, lipoprotein cytochrome c	0.33
GSU2076	OmcZ, cytochrome c	0.28
GSU0618	OmcE, cytochrome c	0.88
GSU2504	OmcS, cytochrome c	0.56
GSU0364	PpcB, cytochrome c	0.98
GSU0592	OmcQ, cytochrome c	0.40
GSU2495	Cytochrome c, 22 heme-binding site	0.20
GSU0837	Response regulator	0.04
GSU0345	nuoH-1, NADH dehydrogenase I	0.74

GSU2813	CcpA, cytochrome c peroxidase	0.35
GSU0615	Cytochrome c, 5 heme-binding site	0.28
GSU2937	Cytochrome c, 5 heme-binding site	0.49
GSU1106	GltA, type I citrate synthase	0.48

n-fold change were calculated based on the 2^{-DDCT} method (21). Avg= Average; -Pd= without palladium and +Pd= with palladium.

FIGURES

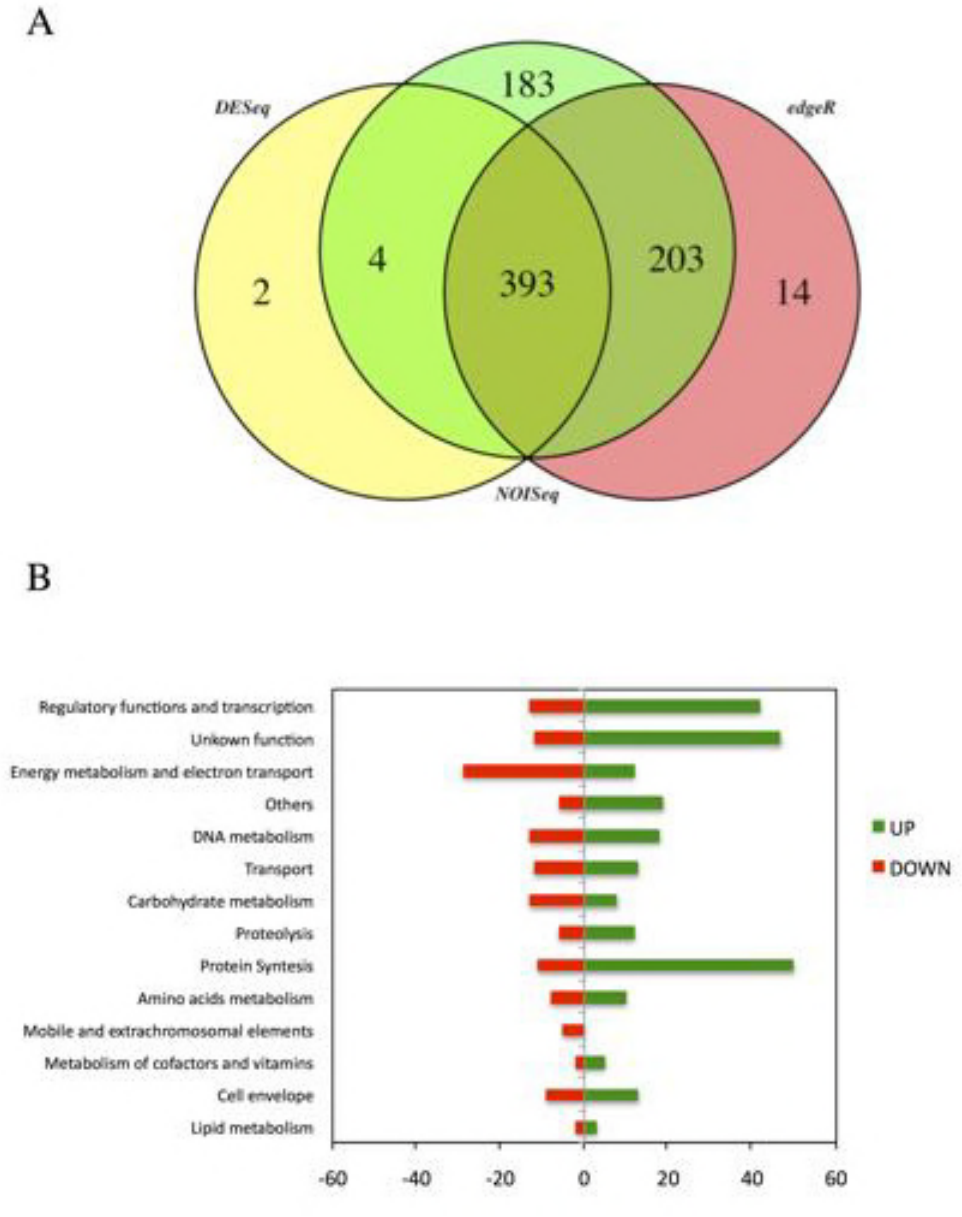

FIG 1. Transcriptome analysis results from *Geobacter sulfurreducens* under Pd(II)-reducing conditions. (A) Venn diagram representing differential gene expression analysis from Pd(II)-reducing conditions by three statistical methods. (B) Functional overview of the genes that were differentially expressed during Palladium reduction.

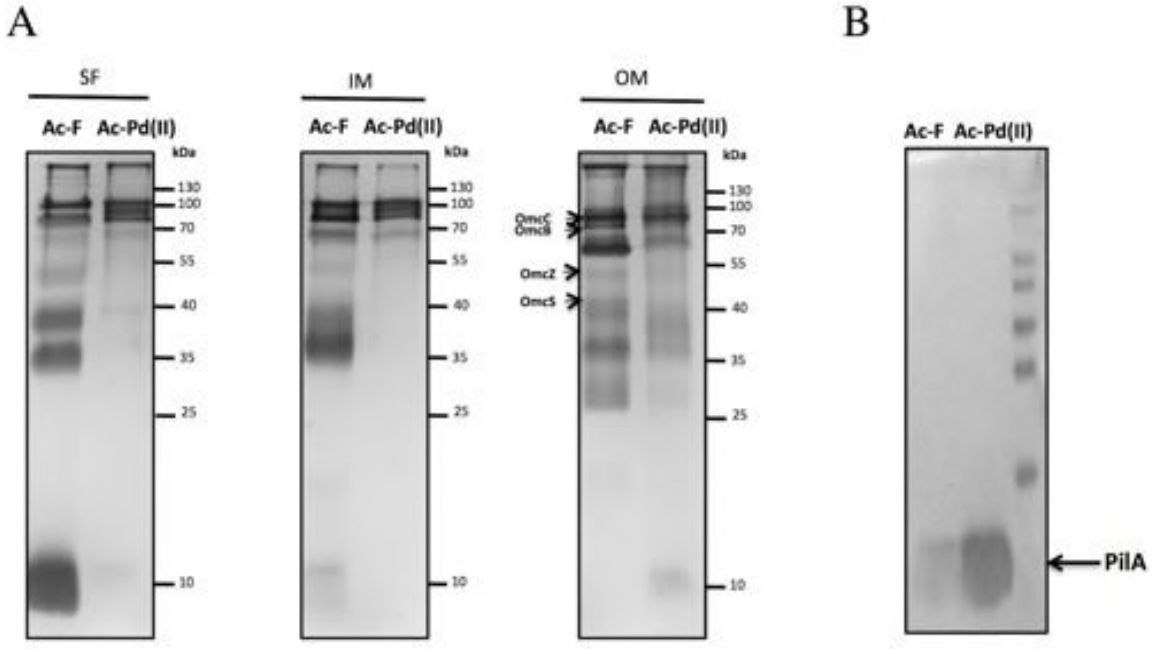

FIG 2. *c*-type cytochrome and PilA protein content found under Pd(II)-reducing conditions. (A) SDS-PAGE heme stained. Outer membrane (OM), inner membrane (IM) and soluble fraction (SF) were prepared from PCA strain with acetate-fumarate (Ac-F) or acetate-Pd(II) (Ac-Pd(II)). The localization of OmcC, OmcB, OmcS, OmcZ and PpcA were labeled based on expected molecular weight (78.96, 74.89, 42.94, 47.09 and 7.72 kDa, respectively). (B) Immunoblot analysis for PilA. The PageRuler Pre-stained Protein Ladder standard (ThermoScientific) was used as a molecular weight.

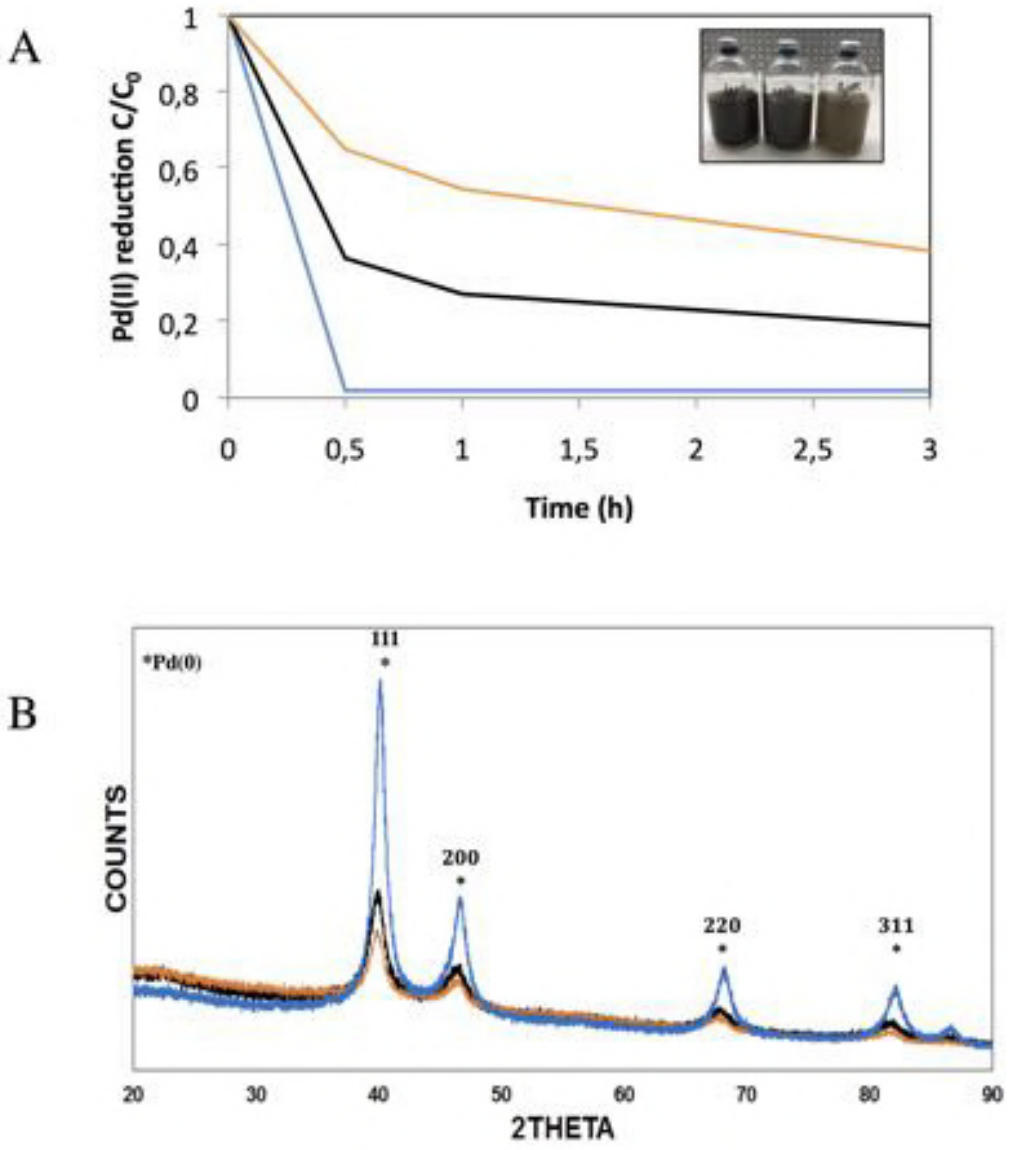

FIG 3. Palladium reduction by different strains of *G. sulfurreducens*. (A) Kinetics of Pd(II) reduction. Photograph of bottles applied for Pd(II) reduction is shown in the inset. (B) Comparison of XRD patters corresponding to cells and black precipitates obtained from cultures of WT, Δ*pilA* and Δ*pilR* strains under Pd(II)-reducing conditions. In (A) and (B), WT strain line blue, Δ*pilA* strain line black, Δ*pilR* strain line orange.

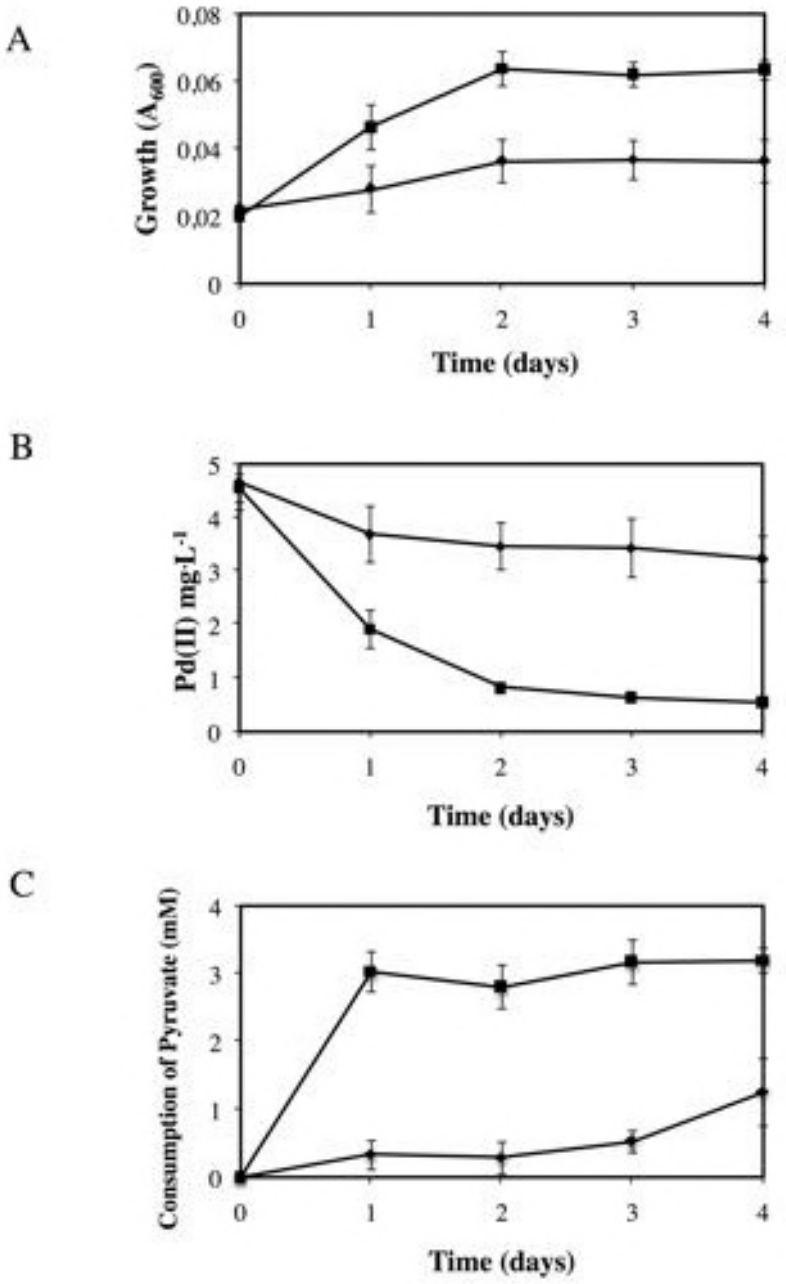

FIG 4. Growth of *Geobacter sulfurreducens* coupled to Pd(II) reduction. (A) Growth. (B) Pd(II) reduction. (C) Consumption of pyruvate. In A, B and C, line with squares, pyruvate-Pd(II) supplemented with glutamine; line with rhombi, pyruvate-Pd(II).

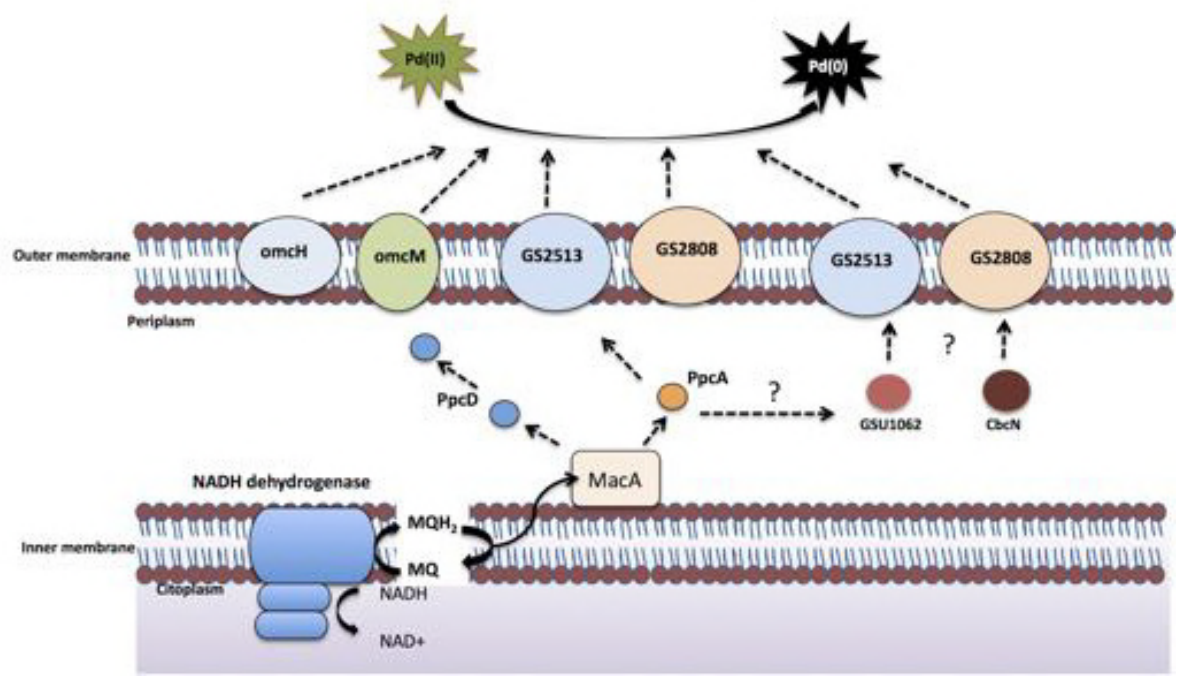

FIG 5. Model of Pd(II) reduction by *Geobacter sulfurreducens*.