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

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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. $\Delta pilA$ and $\Delta pilR$ 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 °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): NaHCO₃, 2.5; NH₄Cl, 0.25; NaH₂PO₄·H₂O, 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 N₂/CO₂ (80:20, v/v). Cell suspensions, as well as acetate and Na₂PdC₄ (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 °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 μ l 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 °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 Na₂PdCl₄ and 15 mM glutamine. Cultures were incubated at 30 °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 H₂SO₄ 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).

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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 *glcA* (citrate

synthase), *nouA* (NADH dehydrogenase I subunit), *atpG* (ATP synthase FoB'), *srfB* Reductase B' subunit), *icd* (isocitrate dehydrogenase) and *gntR* (35).

We observed a decrease in the expression of *gltA*, *icd-mdh*, *atpG* and *nuo* genes, likely by the upregulation of *hgtR* under Pd(II)-reducing conditions. The increase in *hgtR* expression in our experiments was presumably due to the presence of Pd(II), and not to hydrogen, because we used strict anaerobic conditions under a N₂/CO₂ (80/20%) atmosphere without H₂. In addition, *nuoH-1*, *nouD*, *nuoL-1* and *nuoF-1*, which form part of an operon of 14 genes that encode for the NADH dehydrogenase, were also downregulated (see Table S1).

The transcriptional regulator Fur was also upregulated during Pd(II) reduction. Fur activity is controlled by intracellular levels of Fe(II) and directly regulates the expression of the *feoAB*, *gsu2432*, *gsu2937*, *gsu3274*, *ideR* and *gsu1639* genes (36). However, the regulon Fur also represses genes, such as citrate synthase (*gltA*), isocitrate dehydrogenase (*icd*), 2-oxoglutarate oxidoreductase (*gsu1467*, *gsu1468*, *gsu1469* and *gsu1470*), malate dehydrogenase (*mdh*), NADH (*gsu0346*, *gsu0347*, *gsu0348*, *gsu0349*, *gsu0350*, *gsu0351*) and OmcZ (*gsu2076*) (36). Fur upregulation during Pd(II) reduction may be the result of iron limiting condition, so that cells would have been trying to overexpress genes related to homeostasis and iron uptake. With these results, we can observe that during Pd(II) reduction, *gltA*, *icd*, *mdh*, *gsu1466*, *gsu1468*, *gsu0349* and *omcZ* were downregulated and could be the result of Fur and HgtR overexpression.

Among the genes coding for possible transcriptional regulators that are upregulated during Pd(II) reduction are *gsu1072*, *gsu0922*, *gsu1639*, *gsu2980*, *gsu3206*, *gsu2185* whose 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, Immunoblotting 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 $\Delta pilR$ 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 $\Delta pilA$ and $\Delta pilR$ 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 $\Delta pilA$ strain, 81% of Pd(II) was reduced during the same incubation period. When we tested the $\Delta pilR$ 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.

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

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 *glcA* (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.

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

Validation of selected differentially expressed genes using RT-qPCR

To verify results obtained from RNA-Seq experiments and to get quantitative data to compare the transcript abundances under Pd(II)-reducing conditions, we performed RT-qPCR analyses from 24 selected genes encoding proteins involved in electron transfer, transcriptional regulators and central metabolism (Table 4). These genes included the *c*-type cytochromes genes (*omcH*, *omcM*, *omcB*, *omcC*, *omcS*, *omcZ*, *ppcD*, *gsu1062*, *ppcB*, *omcE*, *ccpA*, *gsu0615*, *gsu2937*, *gsu2513*, *gsu2808*, *omcQ* and *gsu2495*) and the cold shock DNA/RNA-binding protein, *gsu0207*, the transcriptional regulators, *hgtR* and *gsu0837*, the menaquinol oxidoreductase complex Cbc3, *gsu1650*, the pilin protein, *pilA*, the NADH dehydrogenase I, *nuoH-1*, and the citrate synthase I, *gltA*.

Upregulation of *omcH*, *omcM*, *gsu1062*, *gsu2513*, *gsu2808* and *ppcD* genes that encoded for *c*-type cytochromes under Pd(II)-reducing conditions was confirmed by RT-qPCR. Similarly, the expression of *pilA*, *gsu0207*, *hgtR* y *gsu1650* was high under these conditions according to the RT-qPCR. On the other hand, the low transcription of *omcB*, *omcC*, *omcS*, *omcZ*, *gsu0837*, *gsu0345*, *gsu2813*, *gsu0615*, *gsu2937*, *omcQ*, *gsu2495* and *gsu1106* observed in RNA-Seq analyses was confirmed by RT-qPCR. Furthermore, the low transcription of *nuoH-1* and *gltA* genes was also observed, in agreement with the high expression of *hgtR*, a negative regulator for these genes (35).

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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Figure Legends

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 patterns corresponding to cells and black precipitates obtained from cultures of WT, $\Delta pilA$ and $\Delta pilR$ strains under Pd(II)-reducing conditions. In (A) and (B), WT strain line blue, $\Delta pilA$ strain line black, $\Delta pilR$ 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*.

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

Strain	Description	Reference/source
<i>Geobacter sulfurreducens</i>		
PCA strain	Wild type	4
$\Delta pilR$ strain	<i>pilR</i> ::kan, kanamycin resistance	23
$\Delta pilA$ strain	<i>pilA</i> ::kan, kanamycin resistance	12
Oligonucleotides	Sequence 5' → 3'	
qPCRrecCfw	CTGTCGTCACCCTTTGTTCC	This study
qPCRrecCrev	GAAAGGGATAGGAGCCGTTTC	This study
qPCRomcMfw	TGGAGACTACCCATGCTGAA	This study
qPCRomcMrev	AGACGTCGAGGTGCTCGTAT	This study
qPCRomcHfw	ATGGACGTGAATGGAAGGAG	This study
qPCRomcHrev	TGGCAGTCAGTACAGGTGGA	This study

qPCRppcDfw	CAGCACTCACCTGTTCTGT	This study
qPCRppcDrev	TGCTTTTTGTGGTCGAAGGT	This study
qPCRhgtRfw	GAGAGAAAATCCGCGGTACA	This study
qPCRhgtRrev	TAGCCTCCCTCATGATGTCC	This study
qPCRGSU1062fw	TCCAGGATTCCGAAACTCAA	This study
qPCRGSU1062rev	CGGCTCTATTTGCTCTCAG	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	AGTTCCCCAGTACGTGTTTCG	This study
qPCRGSU1106rev	GCGAACTTGGAATCCTTTTG	This study
qPCRomcBfw	GGAGTATGTGGCATCCCTTG	This study

qPCRmcBrev	ACCGTTGGCATTTCGTATCTC	This study
qPCRmcCfw	AGAGTACGTGGCATCCCTTG	This study
qPCRmcCrev	CCGTTGGCATTTCGTATCTCT	This study
qPCRmcSfw	TCCTACCAGAACAGCAACGA	This study
qPCRmcSrev	ATAGGAACCGCTCAGGGACT	This study
qPCRmcZfw	AAGCCGACTGTCTCGAGTGT	This study
qPCRmcZrev	CGGAGGTATTGATGCAGCTT	This study
qPCRmcEfw	CCAGATCTGCGTGTCTGTC	This study
qPCRmcErev	CATGCTGCTGGACGAGTAGA	This study
qPCRppcBfw	CCACAAGAAACACCAGACGA	This study
qPCRppcBrev	CACCCCTTGCAGGACTTG	This study
qPCRpilAfw	AATTACCCCCATACCCAAC	This study
qPCRpilArev	AGCAGCTCGATAAGGGTGAA	This study
qPCRGSU2513Fw	GACCAGGCCAGTTCAAGTA	This study
qPCRGSU2513Rv	GGTGTCGATTTCAATTCCTG	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	Fold	log2 FC	P-value	Signal	Cellular location
		name	change			peptide	
GSU0612	Cytochromec3, 3 heme-binding sites	<i>ppcA</i>	0.381	1.390	1.4E-06	Yes	Periplasmic
GSU1024	Cytochromec3, 3 heme-binding sites	<i>ppcD</i>	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	<i>cbcN</i>	0.476	1.071	2.6E-04	Yes	Periplasmic
GSU3107	Ribosomal protein L31, 1 heme-binding site	<i>rpmE</i>	0.404	1.307	5.5E-03	NO	Cytoplasmic
GSU2883	Cytochrome c family protein, 18 heme-binding sites	<i>omcH^a</i>	0.789	0.342	1.0E+00	Yes	Outer membrane
GSU2294	Cytochrome c family protein, 4 heme-binding sites	<i>omcM^a</i>	0.530	0.917	5.8E-02	Yes	Outer membrane

Cytochromes genes that were downregulated in RNA-Seq experiments

Locus ID	Gene annotation	Gene	Fold	log2 FC	P-value	Signal	Cellular location
		name	change			peptide	
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	<i>omcQ</i>	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	<i>omcZ</i>	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	<i>omcC</i>	2.125	-1.088	2.3E-03	Yes	Outer membrane
GSU2737	Polyheme membrane-associated cytochrome c, 8 heme-binding sites	<i>omcB</i>	4.047	-2.017	4.6E-02	Yes	Outer membrane
GSU2811	Cytochrome c Hsc, 1 heme-binding site	<i>cccA</i>	2.657	-1.410	6.8E-16	Yes	Periplasmic
GSU2813	Cytochrome c551 peroxidase, 2 heme-binding sites	<i>ccpA</i>	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	<i>omcS</i> ^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

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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	<i>dksA</i>	0.324	1.63	5.7E-05
GSU0298	Two-component system	<i>fgrM-N</i>	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	<i>scdA</i>	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	<i>gnfK</i>	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	<i>fur</i>	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	<i>ihfA-1</i>	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	<i>glnB</i>	0.094	3.41	1.0E-16
GSU1999	RNA-binding protein Hfq	<i>hfq</i>	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	<i>gnfR</i>	0.055	4.19	4.5E-06
GSU2980	CopG family transcriptional regulator, nickel-responsive regulator	<i>nikR</i>	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	<i>hgtR</i>	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	<i>iscR-1</i>	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	<i>rnk-2</i>	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	<i>ihfB-2</i>	3.979	-1.99	1.1E-02
GSU2815	Sensor histidine kinase, PAS domain-containing	-	2.419	-1.27	3.3E-02

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

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

666

667 n-fold change were calculated based on the 2^{-DDCT} method (21). Avg= Average; -Pd=

668 without palladium and +Pd= with palladium.

669

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672

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 patterns corresponding to cells and black precipitates obtained from cultures of WT, $\Delta pilA$ and $\Delta pilR$ strains under Pd(II)-reducing conditions. In (A) and (B), WT strain line blue, $\Delta pilA$ strain line black, $\Delta pilR$ 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*.