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A Novel Aerobic Mechanism for Reductive Palladium Biomineralization and Recovery by *Escherichia coli*

Joanne M. Foulkes[a], Kevin Deplanche[b], Frank Sargent[c], Lynne E. Macaskie[b], and Jonathan R. Lloyd[a]

[a]Williamson Research Centre for Molecular Environmental Science and School of Earth, Atmospheric and Environmental Sciences, University of Manchester, Manchester, United Kingdom; [b]Unit of Functional Bionanomaterials, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom; [c]Division of Molecular Microbiology, College of Life Sciences, University of Dundee, Dundee, Scotland, United Kingdom

ABSTRACT

Aerobically grown *E. coli* cells reduced Pd(II) via a novel mechanism using formate as the electron donor. This reduction was monitored in real-time using extended X-ray absorption fine structure. Transmission electron microscopy analysis showed that Pd(0) nanoparticles, confirmed by X-ray diffraction, were precipitated outside the cells. The rate of Pd(II) reduction by *E. coli* mutants deficient in a range of oxidoreductases was measured, suggesting a molybdoprotein-mediated mechanism, distinct from the hydrogenase-mediated Pd(II) reduction previously described for anaerobically grown *E. coli* cultures. The potential implications for Pd(II) recovery and bioPd catalyst fabrication are discussed.

Introduction

The microbial reduction of metals and radionuclides has attracted much interest, as it can be potentially harnessed for bioremediation, metal recovery, the fabrication of novel nano-biominerals and even energy generation in biobatteries (Lloyd 2003; Lloyd et al. 2008; Lovley 2006). For example, the sulfate-reducing bacterium (SRB) *Desulfovibrio desulfuricans* has been shown to use a periplasmic hydrogenase supplied with hydrogen to reduce soluble Pd(II), resulting in the precipitation of Pd(0) nanoparticles in the periplasm of the cell ('bioPd'). However SRB produce H_2S, a potent catalyst poison that must be removed before making the bioPd.

Other organisms capable of this metal bioreduction include the Gram-negative bacteria *Shewanella oneidensis* (De Windt et al. 2005), *Escherichia coli* (Deplanche et al. 2010, 2014; Mabbett et al. 2006), *Pseudomonas putida, Cupriavidus necator* (Søbjerg et al. 2009), *Cupriavidus metallidurans* (Gauthier et al. 2010), *Paracoccus denitrificans* (Bunge et al. 2010), *Rhodobacter sphaeroides* (Redwood et al. 2008), *Rhodobacter capsulatus* (Wood et al. 2010), and the Gram-positive bacteria *Bacillus sphaericus* (Creamer et al. 2007), *Arthrobacter oxidans* (Deplanche et al. 2014; Wood et al. 2010), *Micrococcus luteus* (Deplanche et al. 2014), *Staphylococcus sciuri* (Søbjerg et al. 2009) and *Clostridium pasteurianum* (Chidambaram et al. 2010).

This property has allowed the use of "palladized" whole cells or processed biomineral directly in industrially important reactions, often showing superior activity compared with a commercially available carbon-supported palladium catalyst. A number of studies have investigated the catalytic activity of bioPd, demonstrating its use in remediative reactions such as the reduction of Cr(VI) to Cr(III) (Beauregard et al. 2010; Mabbett et al. 2006), the dehalogenation of chlorophenol, polychlorinated biphenyls, polybrominated diphenyl ethers (Baxter-Plant et al. 2003; De Windt et al. 2005; Harrad et al. 2007), trichloroethylene (Hennebel et al. 2009a, 2009b), and the pesticide γ- hexachlorocyclohexane (Mertens et al. 2007), in "greener" chemical synthesis such as the hydrogenation of itaconic acid (Creamer et al. 2007) and 2-pentyne (Bennett et al. 2010), in Heck and Suzuki reactions (Bennett et al. 2013; Deplanche et al. 2014), and also in the application of bioPd as a fuel cell electrocatalyst to produce electricity from hydrogen (Orozco et al. 2010; Yong et al. 2007). In each case where the bioPd was compared with an abiotically produced palladium catalyst (finely divided or supported on a carbon matrix), the bioPd was more active than or at least as active as the commercially available alternative.

Production of catalytically active bioPd also was reported by an aerobically grown *Serratia* sp. (Beauregard et al. 2010; Deplanche et al. 2014) under which condition hydrogenases are not expressed. Also, cells of *E. coli* deficient in the three major hydrogenases reduced Pd(II) (albeit slowly: Deplanche et al. 2010), and showed larger Pd-nanoparticles located on the outer surface of the cells. This suggested an alternative mechanism of Pd(II) reduction which has not been investigated.

E. coli produces bioPd which is comparably active to that produced by *D. desulfuricans* (Deplanche et al. 2014). This also provides a very useful model organism since it is facultatively anaerobic and has well-defined molecular tools to elucidate

reaction mechanisms under aerobic and anaerobic conditions. The enzymes potentially involved in the bioreduction of palladium by *E. coli* under the latter conditions are the nickel-dependent hydrogenase enzymes Hyd-1, Hyd-2, and Hyd-3, and the formate dehydrogenase molybdoenzymes FDH-N, and FDH-H. Another molybdoenzyme, FDH-O, is expressed under both aerobic and anaerobic conditions.

A possible role for FDH-O is to allow bacteria to adapt rapidly to a sudden shift from aerobic respiration to anaerobiosis, before FDH-N has been produced in sufficient amounts to continue formate metabolism (Abaibou et al. 1995). Hyd-1, Hyd-2, FDH-O, and FDH-N are membrane-bound and periplasmically oriented, whereas Hyd-3 and FDH-H are subunits of the formate hydrogenlyase (FHL) complex, an intracellular enzyme complex that is also membrane-bound but which faces into the cytoplasm. The mechanisms responsible for the formate-dependent bioreduction by anaerobically grown cultures of *E. coli* have been studied, showing that the hydrogenase enzymes Hyd-1 and Hyd-2 are mainly responsible for Pd(II) bioreduction (Deplanche et al. 2010). In a study of formate-dependent Pd(II) bioreduction by *Desulfovibrio fructosovorans*, the deletion of the periplasmic hydrogenases caused the Pd(0) nanoparticles to be relocated to the cytoplasmic membrane site of the remaining hydrogenases, indicating that the periplasmic hydrogenases are at least partially involved (Mikheenko et al. 2008).

The growth yield of anaerobic cultures is lower than that of aerobic cultures, and for economic production at scale a method of growth of high biomass density is required. When using anaerobic cultures there is also the cost of supplementing with sodium fumarate and glycerol. The dual aims of this study are to establish whether *E. coli* cells grown aerobically are capable of manufacturing bioPd and to identify the enzyme(s) responsible for such metal reduction. A move away from the need for anaerobic growth would simplify the preparation of high levels of active biomass for catalyst production at industrial scale.

Methods

Bacterial growth

Starter cultures: 50 ml LB broth in a 500-ml Erlenmeyer flask was inoculated with a single isolated colony of the *E. coli* strain under investigation and incubated aerobically (37°C, shaking at 180 rpm for 18 h).

Aerobic cultures

An 11-ml starter culture was added to 99 ml LB broth in a 1-L Erlenmeyer flask. Flasks were incubated for 24 h (37°C, 180 rpm) to produce stationary phase 'resting' cells. The pH of the cells after 24-h incubation was measured to determine that organic acids had not been produced that would otherwise lower the pH considerably (Vasala et al. 2006). Oxygen saturation of a 5-ml aliquot of the broth culture was measured immediately after 24 h of incubation using an Oakton D06 Acorn Series dissolved oxygen meter.

Reduction of Pd(II) to produce bioPd on bacteria

The aerobically grown liquid culture was divided between two 50-ml Falcon tubes and washed three times in 20 ml MOPS-NaOH (morpholinepropanesulfonic acid) buffer, 20 mM at pH7.6 after centrifugation for 20 min at 2500 *g*. Cell pellets were adjusted to a mass of 250 mg wet pellet weight, and resuspended in the MOPS-NaOH buffer to a volume of 1 ml. One tube of 250 mg wet weight cells was resuspended in 22.5 ml MOPS-NaOH buffer with 1 mM sodium tetrachloropalladate in a 30 ml bottle sealed with a butyl rubber stopper. The bottle was incubated in the dark at 30°C for 1 h for the Pd(II) to biosorb to the cells (Baxter-Plant et al. 2003). Then 2.5 ml 10 mM sodium formate was then added to the bottle to initiate bioreduction of the Pd(II).

Use of mutants to determine electron transfer pathway to Pd(II)

In order to investigate the possible role of the aerobic formate dehydrogenase (FDH-O) and other hydrogenase/formate dehydrogenase enzymes in the reduction of Pd(II) by aerobically grown cells of *E. coli*, the rates of reduction by six different additional strains (Table 1) were compared by measuring the Pd(II) remaining in solution by ICP-MS. The strains were "palladized" as above, and rates of reduction/removal compared to those in a series of controls: killed cells (MC4100), cell-free suspension, and live cells (MC4100) unsupplemented with formate.

All strains except BL21(DE3) were from the culture collection of Professor Frank Sargent at the College of Life Sciences, University of Dundee. Strain BL21(DE3) was obtained from Invitrogen, Paisley, UK. Strain MC4100 Δ*moaA* was created by disruption of the *moaA* gene, which encodes the molybdenum cofactor biosynthesis protein A, using the method of Datsenko and Wanner (2000) whereby PCR products are used to disrupt the gene of choice by recombination using the plasmid-borne phage λ Red recombinase.

X-ray diffraction (XRD) analysis

The black precipitates were washed once in acetone and air dried before analysis by X-ray diffraction (XRD). The

Table 1. *E. coli* strains used to determine biological involvement in the reduction of palladium (II) using formate as the electron donor.

Strain	Genotype	Phenotype	Reference
BL21(DE3)	F2 *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*])	Wild type strain commonly used for recombinant protein expression.	(Studier and Moffatt 1986)
MC4100	F- Δ*lacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301*	Parental strain for FTD128 and Δ*moaA*.	(Casadaban and Cohen 1979)
BW25113	*lacI*^{q} *rrbB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsd*R514 Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Parental strain for JW2682 and JW3865.	(Datsenko and Wanner 2000)
FTD128	As MC4100, with in-frame deletion in the *fdhE* gene.	FDH-O & FDH-N negative.	(Luke et al. 2008)
JW2682	As BW25113, with in-frame deletion of the *hypF* gene.	Deficient in all hydrogenases.	(Baba et al. 2006)
JW3865	As BW25113, with in-frame deletion of the *fdoG* gene.	FDH-O negative.	(Baba et al. 2006)
MC4100 Δ*moaA*	As MC4100, disruption of the *moaA* gene.	Deficient in all molybdoenzymes	This study

measurements were performed on a Bruker D8 Advance diffractometer, using Cu κ-α1 radiation. The samples were scanned from 5–70 degrees 2θ in steps of 0.2 degrees, with a count time of 2 sec per step.

Extended X-ray absorption fine structure (EXAFS)

Aliquots of the cell/Pd/formate suspension were taken at times 0 and 30 min, and 1, 3 and 4 h from the addition of formate, and frozen immediately in liquid nitrogen. The direct reduction of Pd(II) to Pd(0) was demonstrated using EXAFS, performed at the European Synchrotron Radiation Facility (ESRF), in Grenoble, France. The samples were transported to the synchrotron at ESRF on dry ice, where they were thawed and injected immediately into sample holders, before freezing once more in liquid nitrogen and placing into the beam. X-ray absorption data were collected on beamline BM29 at the Pd K–edge in the energy range 24,200–24,900 eV.

Data were recorded at low temperature (77 K) and under vacuum to reduce the thermal Debye-Waller factor and prevent oxidation. A Si(III) double crystal monochromator was used, calibrated with a Pd foil, and the spectra were collected in fluorescence mode using a 13-element solid-state detector. A reference spectrum of a palladium foil was recorded in transmission mode on station 9.3 at the SRS Daresbury. The data were background subtracted and the EXAFS spectra fitted in DL Excurv (http://www.cse.scitech.ac.uk/cmg/EXCURV/) using full curved wave theory (Gurman et al. 1984).

Transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS)

Following Pd(II) reduction, cells were stored at 10°C overnight. The cell pellets were then rinsed twice with deionized water, fixed in 2.5% (wt/vol) glutaraldehyde, centrifuged for 5 min at 16,000 g, resuspended in 1.5 ml of 0.1 M cacodylate buffer (pH 7) and stained in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7 (60 min). Cells were dehydrated using an ethanol series (70, 90, 100, 100, 100% dried ethanol, 15 min each) and washed twice in propylene oxide (15 min, 9500 g). Cells were embedded in epoxy resin and the mixture was left to polymerize (24 h; 60°C). Sections (100–150-nm thick) were cut from the resin block, placed onto a copper grid and viewed with a

JEOL 1200CX2 TEM, accelerating voltage 80 keV. EDS was performed on electron-dark areas, to confirm the presence of palladium.

Results

Palladization of E. coli BL21(DE3)

The pH of the aerobically grown liquid culture was between 7.7–7.9, indicating that there was not extensive production of organic acids due to overflow metabolism. Oxygen saturation measurements showed that the liquid culture was 72% saturated following 24 h of incubation, indicating that it was not oxygen-limited. After harvesting, the cells were able to couple the reduction of Pd(II) to the oxidation of formate, indicated by the rapid formation of a black precipitate, tentatively identified as Pd(0) (Figure 1). ICP-MS analysis confirmed complete removal of Pd(II) from solution within 45 min, and the presence of crystalline Pd(0) was confirmed using XRD in this, but not in the heat-killed cells control where the cells removed substantial Pd(II) abiotically. An increase in metal biosorption by heat killed biomass as compared to live cells is well documented (Machado et al. 2009; Parameswari et al. 2009) and was attributed to loss of membrane integrity to reveal additional intracellular metal binding sites (Machado et al. 2009).

Extended X-ray absorption fine structure (EXAFS)

The nature of the Pd associated with the biomass was assessed further using X-ray absorbance spectroscopy. The features in the corresponding EXAFS spectra (Figure 2) are due to the wavelike nature of the photoelectron, which is released from the atom with increasing energy and scattered from surrounding atoms with new waves being emitted. With increasing photon energy, the interference between the waves alternates between constructive and destructive, which leads to oscillations in the spectrum. Examining these oscillations gives information on the number, species and distance of the surrounding atoms. As seen in Figure 2, the samples taken at times 0 and 30 min, which contain Pd(II), have identical EXAFS spectra. The samples taken at 60 min onwards are identical to the Pd(0) foil control, which indicates that only Pd(0) was present.
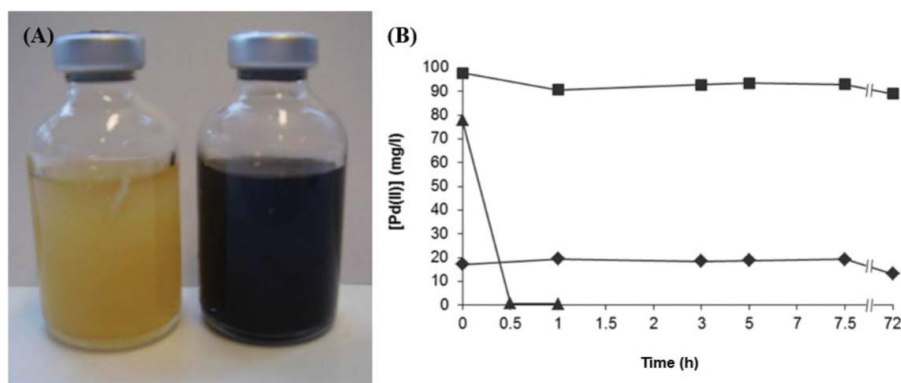

Figure 1. (A) Complete reduction of Pd(II) to Pd(0) by an aerobically grown culture of E. coli. Both bottles contain cells resuspended in 20 mM MOPS buffer at pH 7.6, and 1 mM sodium tetrachloropalladate (total volume 25 ml). This image was taken 45 min after the addition of formate to the bottle on the right. (B) Reduction by E. coli MC4100 and by controls showing no abiotic reduction of Pd(II). Controls used were killed (autoclaved) cells and cell-free suspension. Soluble Pd(II) in the supernatant was measured using ICP-MS. ▲ = MC4100; ■ = no cells; ◆ = killed cells.
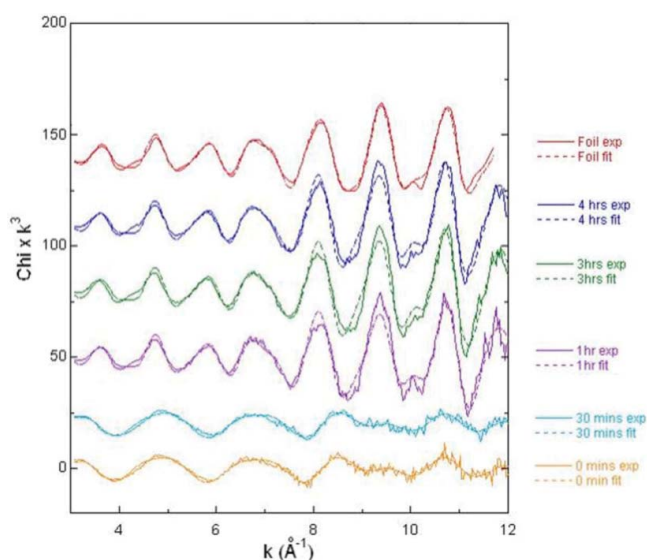
Figure 2. EXAFS data showing the presence of Pd(II) at 0 and 30 min (bottom two traces), and Pd(0) at 1, 3 and 4 h (ascending series). The top trace is palladium foil.

Reduction of the Pd(II) to Pd(0) was therefore confirmed to be complete in less than 30 min, as confirmed by ICP-MS analysis.

Use of mutants to determine electron transfer pathway to Pd(II)

Aerobic cultures of the parental strains MC4100 and BW25113 and the strain which lacked all hydrogenases (JW2682) removed Pd(II) identically with no residual Pd(II) detected after 30 min (Figure 3). Removal of the hydrogenase enzymes had no effect on the rate of palladium removal from solution, confirming that these hydrogenases have no role in the aerobic reduction of Pd(II). The FDH-O-negative strain JW3865 reduced Pd(II) within 1 h, and the FDH-O/FDH-N-negative strain FTD128 within 2 h. Strain MC4100 $\Delta moaA$, lacking all molybdoenzymes, reduced the palladium within 7 h.

These results indicate the likely involvement of the FDH-O enzyme in the reduction of Pd(II) by aerobically grown *E. coli* using formate, although other Mo-containing enzymes must
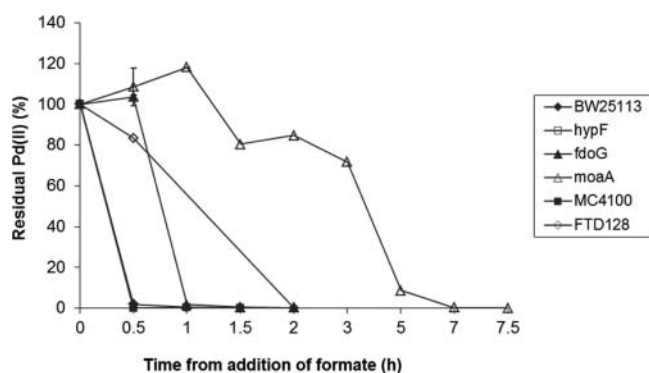

Figure 3. Pd(II) reduction by six different strains of *E. coli*, using formate as the electron donor. Soluble Pd(II) in the supernatant was measured using ICP-MS. ◆ = BW25113; □ = JW2682; ▲ = JW3865; △ = MC4100 $\Delta moaA$; ■ = MC4100; ◇ = FTD128. Data points for BW25113, JW2682 and JW3865 are mean values of triplicates, with standard error shown.

also be involved given the impaired metal reduction noted with the Δmoa mutant. Controls containing no biomass showed no abiotic reduction of Pd(II) using formate (Figure 1B), although a brown precipitate was seen in the no-formate control. The X-ray powder diffraction pattern did not show the presence of any peaks characteristic of Pd(0) in this precipitate, indicating that it was probably amorphous and noncrystalline. Time zero on Figure 3 is the point at which formate was added, following 1 h of incubation to allow biosorption of the Pd(II) to the cells; hence the abiotic Pd(II) removal by killed cells (Figure 1) was apparent at the time of formate addition with no evidence for further Pd(II) reduction.

Transmission electron microscopy (TEM)

TEM images of thin sections of cells showed that with all strains the reduced palladium was precipitated predominantly in the extracellular matrix of the cultures (Figure 4), although it appears that the nanoparticles may be associated with the outer membrane of the cells. Energy dispersive X-ray spectroscopy (EDS) confirmed the presence of palladium in these precipitates.

Discussion

The results from this study demonstrate that it is possible for aerobically grown cultures of *E. coli* to reduce Pd(II) enzymatically, with no need to remove oxygen from the experimental system during the bioreduction step. Autoclaved control experiments indicate that Pd(II) bioreduction in these cultures is enzymatic, with reduction of palladium not occurring in the absence of viable cells irrespective of the length of incubation. The major enzymes shown to be involved include the formate dehydrogenases FDH-O and FDH-N, although bioreduction still occurs in strains without these enzymes albeit at a much lower rate. Other molybdoenzymes must therefore be involved.

The strain that lacked all molybdoenzymes did however still reduce the palladium, although this took 7 h, compared with less than 30 min by the wild-type strains. Hydrogenases, implicated as the dominant Pd(II) reductases in other experimental systems grown under anaerobic conditions (Deplanche et al. 2010; Mikheenko et al. 2008), are not expressed in aerobically grown cultures, and their lack of involvement was evident as the strain lacking hydrogenase enzymes reduced palladium at the same rate as the wild-type strains in this study.

Furthermore, whichever biological system is responsible for the aerobic bioreduction of Pd(II), there seems to be little impact on the site of Pd(0) deposition. The location of the bio-reduced Pd(0) in our experiments is almost always extracellular, although often associated with the outer membrane of the cells. This is particularly the case with the MC4100 $\Delta moaA$ strain (which lacks all molybdoenzymes), in which the majority of the Pd(0) nanoparticles are closely associated with the outer membrane (Figure 4E).

One conclusion that may be drawn from this is that whilst cells that lack the formate dehydrogenases are still capable of reducing Pd(II), when all of these enzymes are missing a cellular component associated with the outer membrane may be responsible. Furthermore, this formate oxidation activity is
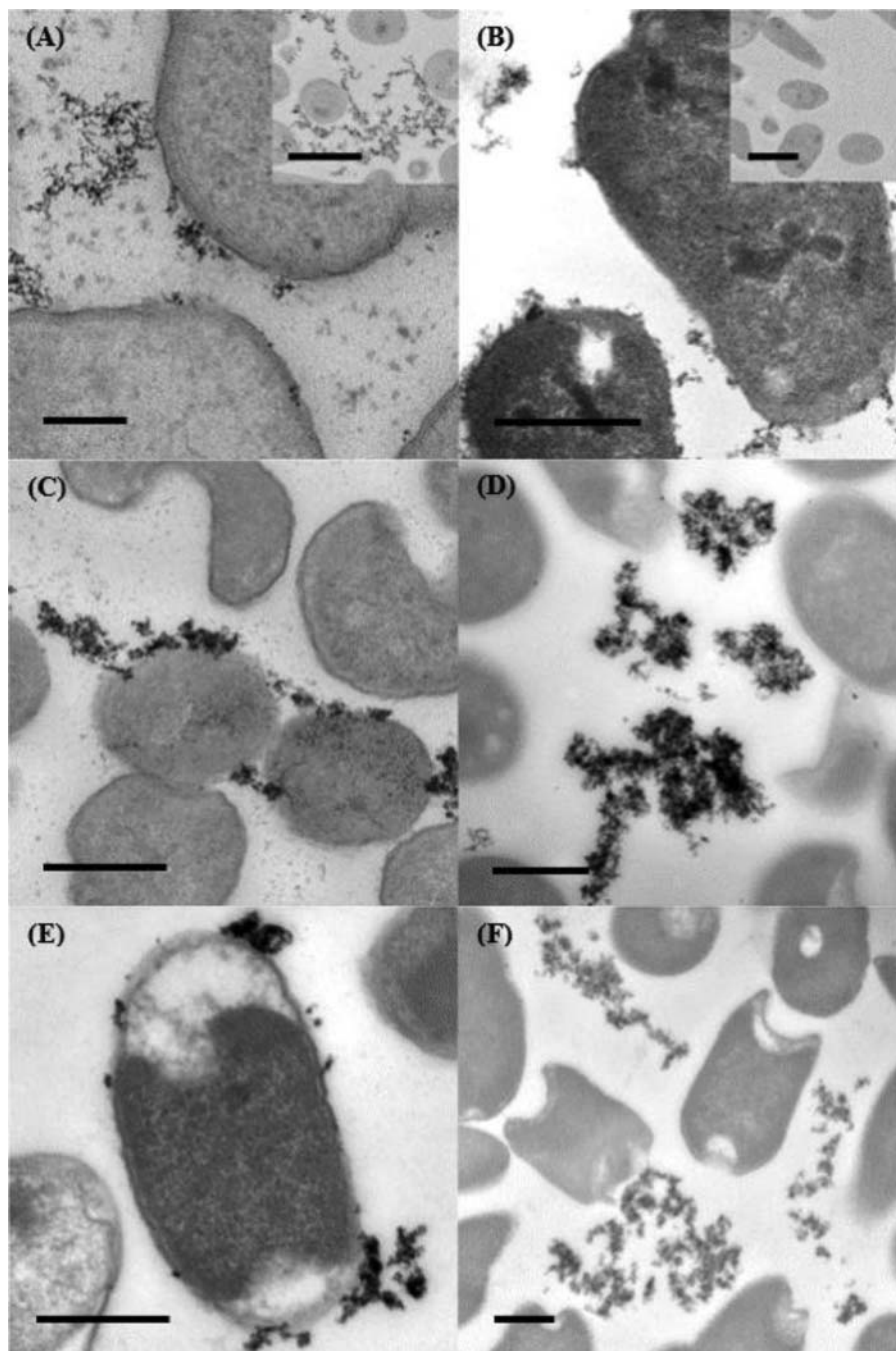
Figure 4. TEM of thin sections of aerobically grown cells showing extracellular palladium; (A) MC4100, inset BL21; (B) BW25113, inset BL21 (no Pd); (C) FTD128; (D) JW2682; (E) MC4100 Δ*moaA*; (F) JW3865. Scale bar (A) = 100 nm; (B)-(F) = 500 nm; insets = 1 μm.

much weaker than that seen with the strains containing for-mate dehydrogenases, where Pd(II) reduction is more rapid. It is possible however that following the initial enzymatic reduc-tion of a small percentage of the Pd(II), the Pd(0) nanoparticles formed may themselves be responsible for catalysing the reduc-tion of the remainder of the Pd(II) (Yong et al. 2002), which would mean that only a minor, initial biological input is required.

Although the formate dehydrogenase enzyme systems impli-cated in Pd(II) bioreduction by *E. coli* are periplasmic, the majority of the reduced Pd(0) precipitates outside the cell. It is possible that an electron shuttle system exists similar to that found in *Shewanella oneidensis* (von Canstein et al. 2008) that is as yet undiscovered in *E. coli*. It is also possible that the first Pd(0) nanoparticles to form breach the outer membrane, and themselves form an electron conduit for further Pd(II) reduc-tion outside the cell. The pH of these experiments is also higher than others where Pd(0) nanoparticles accumulated in the peri-plasm (Redwood et al. 2008), which could indicate the higher biosorption of cationic metal to the outer membrane and extra-cellular polymeric substances, which are then not able to enter the periplasm. The influence of a higher pH in the location of the Pd(0) may be confirmed by the observation that Pd(0) nanoparticles were located on the cell surface of

D. desulfuricans when bioreduction of Pd(II) was performed at pH 7 (Yong et al. 2002).

In conclusion, this study has demonstrated the presence of a novel biological mechanism responsible for the bioreduction of Pd(II) in aerobically grown cultures of *E. coli*, catalyzed mainly by molybdenum-containing enzyme systems. Subsequent studies will investigate the catalytic activity and selectivity of the Pd(0) nanoparticles produced under aerobic conditions in a range of industrially important reactions. If active, this new form of bioPd has the advantage over that produced by anaerobic culture as it is easier to produce at high yield, from increased biomass levels associated with aerobic growth.

There is also no requirement for additional processing steps to remove H$_2$S (produced by SRB systems), and the use of formate instead of hydrogen gas means that the procedure is less hazardous and more controllable. The advantages of this more scalable method of synthesis would need to be considered against any alterations in activity/selectivity of the resulting catalyst (versus synthetic and other bioPds), using a cost-benefit analysis. Importantly, identification of the specific enzymatic process(es) involved in the biomanufacture of bioPd is the first step towards application of the tools of synthetic biology for "designer catalyst" production for specific applications.

In a geomicrobiological context, this study shows that aerobic cells of *E. coli* restrict the deposition of Pd(0) to locations outside the cell. However in both *D. desulfuricans* (grown anaerobically) and *Bacillus benzeovorans* (grown aerobically) intracellular depositions of small Pd-nanoparticles were observed at the expense of both hydrogen and formate (Omajali, Mikheenko, Merroun, Wood, and Macaskie in press) and, notably, were also seen in *E. coli* grown anaerobically (Macaskie, Williams, Priestley, and Courtney unpublished). This raises questions about potential biochemical 'trafficking' pathways of Pd(II), the possibility of Pd(II) efflux by aerobic (but not anaerobic) cells and, following from that, the possibility of biogeochemical cycling of this element.

Acknowledgment

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