Involvement of hydrogenases in the formation of highly catalytic Pd(0) nanoparticles by bioreduction of Pd(II) using *Escherichia coli* mutant strains

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Escherichia coli produces at least three [NiFe] hydrogenases (Hyd-1, Hyd-2 and Hyd-3). Hyd-1 and Hyd-2 are membrane-bound respiratory isoenzymes with their catalytic subunits exposed to the periplasmic side of the membrane. Hyd-3 is part of the cytoplasmically oriented formate hydrogenlyase complex. In this work the involvement of each of these hydrogenases in Pd(II) reduction under acidic (pH 2.4) conditions was studied. While all three hydrogenases could contribute to Pd(II) reduction, the presence of either periplasmic hydrogenase (Hyd-1 or Hyd-2) was required to observe Pd(II) reduction rates comparable to the parent strain. An *E. coli* mutant strain genetically deprived of all hydrogenase activity showed negligible Pd(II) reduction. Electron microscopy suggested that the location of the resulting Pd(0) deposits was as expected from the subcellular localization of the particular hydrogenase involved in the reduction process. Membrane separation experiments established that Pd(II) reductase activity is membrane-bound and that hydrogenases are required to initiate Pd(II) reduction. The catalytic activity of the resulting Pd(0) nanoparticles in the reduction of Cr(VI) to Cr(III) varied according to the *E. coli* mutant strain used for the initial bioreduction of Pd(II). Optimum Cr(VI) reduction, comparable to that observed with a commercial Pd catalyst, was observed when the bio-Pd(0) catalytic particles were prepared from a strain containing an active Hyd-1. The results are discussed in the context of economic production of novel nanometallic catalysts.

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INTRODUCTION

Microbial recovery of Pd via reduction of Pd(II) is emerging as a clean alternative to traditional reclaiming treatments, and the potential advantages of this are numerous. Biomass can be rapidly and cheaply grown in large amounts, and the bioreduction process is sensitive enough to recover metal at parts-per-million (p.p.m.) concentrations, which is often below the economic threshold of traditional recovery methods.

The increasing understanding of the mechanisms by which bacteria reduce or oxidize metallic elements has recently led to the development of innovative processes in various fields of biotechnology, including biomining (Rawlings &

Johnson, 2007), bioleaching (Faramarzi *et al.*, 2004) and bioremediation (Larsson *et al.*, 1988), as well as biorecovery (Kashefi *et al.*, 2001; Konishi *et al.*, 2006; Lloyd *et al.*, 1998). Many applications harness the ability of some bacteria to carry out dissimilatory metal reduction, a process in which the oxidation of simple inorganic or organic substrates (e.g. H₂, formate) is coupled to the reduction of metallic ions by redox-active enzymes (Lloyd *et al.*, 2001; Lovley, 1993). For example, sulphate-reducing bacteria (SRB) such as *Desulfovibrio desulfuricans* have been shown to reduce a wide range of metallic elements (e.g. Fe(III), Cr(VI), Mo(VI), Tc(VII), U(VI)) (Lloyd, 2003) in addition to Pd(II) (Lloyd *et al.*, 1998). De Luca *et al.* (2001) demonstrated the importance of periplasmic [NiFe] hydrogenases in the reduction of Tc(VII) using mutants of *Desulfovibrio fructosovorans*, and initial experiments on Pd(II) reduction by the related *D. desulfuricans* showed similarities in the two metal-reduction mechanisms (Lloyd *et al.*, 1998). Mikheenko *et al.* (2008) established that hydrogenases are involved in Pd(II) reduction in *D. fructosovorans*.

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Abbreviations: bioPd(0), biomineralized Pd(0); chemPd(0), Pd(0) produced by chemical reduction; FHL, formate hydrogenlyase; OFN, oxygen-free nitrogen; SRB, sulphate-reducing bacteria; TEM, transmission electron microscopy; XRD, X-ray powder diffraction.

One major advantage of Pd(II) bioreduction is the production of a subpopulation of discrete, stabilized Pd(0) nanoparticles of ~5 nm diameter (Creamer *et al.*, 2007), which exhibit unusual magnetic properties (Mikheenko, 2004; Macaskie *et al.*, 2005). The catalytic potential of this biomineralized Pd(0) (bioPd(0)), as compared to Pd(0) made via chemical reduction of Pd(II) under H₂ (chemPd(0)), was first demonstrated by Yong *et al.* (2002a) in a simple reaction involving the release of H₂ from sodium hypophosphite. This was followed by the demonstration of other catalytic properties of bioPd(0), including the remediation of metallic pollutants (e.g. Cr(VI)) and xenobiotics (halogenated aromatics and flame-retardants) (Baxter-Plant *et al.*, 2003; De Windt *et al.*, 2005; Humphries *et al.*, 2004; Harrad *et al.*, 2007), hydrogenation (Creamer *et al.*, 2007) and the production of electricity in a fuel cell (Yong *et al.*, 2007). BioPd(0) performed comparably to (chemical catalysis) or better than (fuel cell) a commercial catalyst (5 % Pd on carbon).

The mechanism of Pd(II) reduction by *Escherichia coli* has not yet been reported, but the involvement of the periplasmic hydrogenases of *D. fructosovorans* was previously suggested (Mikheenko *et al.*, 2008). The *E. coli* K-12 genome encodes four hydrogenase isoenzymes (Hyd-1–Hyd-4) but only three of them (Hyd-1, Hyd-2 and Hyd-3) have been biochemically characterized. All three are metalloenzymes that contain Fe–S clusters and an unusual Ni–Fe–CO–2CN cofactor at their active sites and, although they possess physiological directionality, they have the ability to operate reversibly *in vitro* at high partial pressure of H₂ (Sawers, 1994). The cytoplasmic-facing, membrane-bound Hyd-3, encoded by the *hyc* operon, is a component of the formate hydrogenlyase (FHL) complex in which, under fermentative conditions, endogenously produced formate is oxidized to CO₂ and H₂ (Ballantine & Boxer, 1985). Hyd-1 and Hyd-2 are also membrane-bound but are both located on the periplasmic side of the cytoplasmic membrane and are encoded by the *hya* and *hyb* operons, respectively. Hyd-1 and Hyd-2 operate as ‘uptake’ hydrogenases for energy conservation under fermentative (Hyd-1) and anaerobic respiratory (Hyd-2) conditions (Sawers *et al.*, 1985).

In this work, following the timely demonstration of Pd and Au biorecovery technologies (Deplanche *et al.*, 2007; Mabbett *et al.*, 2006), we set out to identify the hydrogenase isoenzyme(s) involved in Pd(II) reduction in *E. coli*. To this end, mutant strains were constructed that lacked specific hydrogenase activities and tested for their ability to reduce Pd(II) to bioPd(0). Rousset *et al.* (2006) showed that ‘redirecting’ Pd(0) to the cytoplasmic membrane of *D. fructosovorans* by removal of periplasmic hydrogenases altered the catalytic activity of the resulting material. Hence the second objective was to evaluate the quality and catalytic properties of the resultant bioPd(0) bioinorganic catalysts derived from each specific hydrogenase deletion mutant of *E. coli* as a precursor of ‘engineered’ metallic bionanocatalysts.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study and their genotypes are listed in Table 1. *E. coli* MC4100 (parent strain) was provided by Professor J. A. Cole, University of Birmingham, UK. In-frame deletions were introduced by first constructing the desired deletion on pBluescript (Stratagene) by PCR-based methods as described previously (Sargent *et al.*, 1999). Deletion alleles were then moved onto the chromosome of MC4100 derivatives using the pMAK705 method of Hamilton *et al.* (1989). Strain IC009 ($\Delta hybC \Delta hycE$) was constructed by moving a $\Delta hycE$ allele into strain FTD67 (Dubini *et al.*, 2002). Strain IC010 ($\Delta hyaB \Delta hycE$) was constructed by moving a $\Delta hycE$ allele into strain FTD22 (Sargent *et al.*, 1999). Strain FTD150 ($\Delta hyaB \Delta hybC \Delta hycE \Delta hyfB-R::Spec^R$) was constructed by first moving a $\Delta hycE$ allele into strain FTD89 (Sargent *et al.*, 1999) to give strain FTD147 ($\Delta hyaB \Delta hybC \Delta hycE$), and then moving the $\Delta hyfB-R::Spec^R$ allele from JRG3621 (Skibinski *et al.*, 2002) by P1 transduction into strain FTD147.

All strains were maintained aerobically at 30 °C on nutrient agar plates. To investigate the effect of hydrogenase deletions on Pd(II) reduction, mutated strains were grown in conditions that allowed maximum expression of their specific hydrogenase. Hence, pre-cultures [10 %, v/v, inoculum from a mid-exponential phase culture grown anaerobically in nutrient broth (NB) no. 2 (Oxoid) containing 50 mM sodium formate] were grown at 37 °C in NB no. 2 under either anaerobic respiratory conditions (MC4100, IC009, IC010, FTD150: NB no. 2 supplemented with 0.4 %, w/v, sodium fumarate and 0.5 %, v/v, glycerol, final concentrations) or fermentative conditions (MC4100, FTD89: NB no. 2 supplemented with 50 mM sodium formate) to maximize hydrogenase expression.

Table 1. Bacterial strains used in this study

Strain	Genotype	Hyd-a*			Reference
		1	2	3	
MC4100	Parental strain	+	+	+	Casadaban & Cohen (1979)
IC009	$\Delta hybC \Delta hycE$	+	–	–	This work
IC010	$\Delta hybB \Delta hycE$	–	+	–	This work
FTD89	$\Delta hyaB \Delta hybC$	–	–	+	Sargent <i>et al.</i> (1999)
FTD150	$\Delta hyaB \Delta hybC \Delta hycE \Delta hyfB-R$	–	–	–	This work

*+, Present; –, defective.

For Pd(II) bioreduction experiments, cultures of *E. coli* were grown as above in 2 l Durham bottles, almost filled with medium and sealed with rubber stoppers. Anaerobic conditions were promoted by degassing the bottles under vacuum for 30 min and bubbling oxygen-free nitrogen (OFN) for 30 min. Mid-exponential phase cultures (OD₆₀₀ 0.5–0.7) were harvested under an anaerobic atmosphere (80% N₂, 10% CO₂, 10% H₂) in a NACS anaerobic cabinet (Don Whitley Scientific) by centrifugation (12 000 g, 15 min), washed three times in 100 ml degassed MOPS/NaOH buffer (20 mM, pH 7.2) and degassed under vacuum/flushed with OFN (two cycles of 20 min) to eliminate residual dissolved H₂. Finally, cells were resuspended in 50 ml of the same buffer and stored at 4 °C as concentrated cell suspensions under OFN until use, usually the next day. Cell concentration (mg ml⁻¹) was determined by reference to a pre-determined OD₆₀₀ to dry weight conversion.

Isolation of total membranes from *E. coli* MC4100 and hydrogenase-deficient mutant FTD150. Preparation of soluble and membrane fractions was carried out as described by Ballantine & Boxer (1985). During the membrane purification steps, all buffers contained 0.003% (w/v) sodium dithionite. Cultures (1 l) of *E. coli* MC4100 ('wild type') and FTD150 (mutant lacking all four hydrogenases) were grown under anaerobic respiratory conditions (as above) at 37 °C to an OD₆₀₀ of 0.5–0.8 and harvested by centrifugation (7000 g, 15 min at 4 °C). Cells were washed twice in 50 mM potassium phosphate buffer (pH 6.8) and resuspended in 50 mM Tris/HCl buffer pH 7.5. Cell breakage was achieved by passing cell suspensions four times through a French pressure cell at a pressure of 18 000 p.s.i. (124 200 kPa) in the presence of 5 mg DNase. Cellular debris was removed by centrifugation (20 000 g, 15 min, 4 °C) and the membrane fractions were obtained by further centrifugation of supernatants (130 000 g, 2 h, 4 °C). Supernatants were retained as they contained the cytoplasmic and soluble periplasmic proteins. Membrane pellets were resuspended in 1 ml cold Tris/HCl buffer (pH 7.5) using a syringe to avoid frothing and washed once in the same buffer before being stored under OFN at –80 °C until use, usually the next day. Protein concentration in the membrane fraction was ~30–40 mg ml⁻¹ as determined by the BCA method (Sigma protein test kit TPRO 562, Sigma-Aldrich).

Pd(II) solution. For Pd(II) reduction by soluble and membrane fractions, an aqueous solution of tetraaminepalladium(II) chloride monohydrate (Pd(NH₃)₄Cl₂·H₂O) was used (pH 7.2). For bioreduction studies using whole cells, Pd(II) solution (2 mM) was made by dissolving an appropriate amount of sodium tetrachloropalladate (Na₂PdCl₄) in 0.01 M HNO₃, pH 2.3, as this was previously shown to be optimal for production of active catalyst using whole cells (Yong *et al.*, 2002b).

Bioreduction of Pd(II). For bioreduction studies using whole cells, 40 ml of a freshly prepared Pd(II) solution (initial pH 2.3) was degassed (10 min) and sparged with OFN (10 min). For each strain a suspension of resting cells in 20 mM MOPS/NaOH buffer (pH 7.2) was added to the solution to give a mass ratio of 2:1 dry biomass:Pd(II). The cell/Pd mixture (pH 2.4) was incubated (30 min, 30 °C) with occasional shaking to allow initial biosorption of Pd(II) (as [PdCl₄]²⁻) to cell-surface groups. Pd(II) reduction was initiated by the addition of sodium formate (final concentration 20 mM). Timed samples (1 ml) were withdrawn, centrifuged (12 000 g, 4 min; IEC Centra M bench centrifuge) and supernatants were assayed as described below to estimate the residual Pd(II) concentration. The reduction of Pd(II) by formate using heat-killed cells (15 min; 80 °C) and by cell-free solution (chemical Pd(II) reduction by formate) were used as controls.

Assay of Pd(II). Removal of Pd(II) from test solution was monitored using the spectrophotometric method of Dasages (1978). The Sn(II)

reagent was made by dissolving 29.9 g SnCl₂ powder into 500 ml concentrated HCl. For Pd(II) assay, 200 µl sample was added to 800 µl SnCl₂ solution in a 1.5 ml plastic cuvette and the absorbance at 463 nm was determined after 1 h of incubation at 30 °C against a blank prepared in the same way. The system obeyed Beer's law over the range 5–80 p.p.m. Pd(II). The assay method was validated by analysis of reference and selected test samples by a commercial laboratory (H2b, Capenhurst, UK).

Reduction of Pd(II) using cytoplasmic and membrane fractions.

Degassed Pd(II) solution (20 µl, from the amine salt, pH 7.2, final concentration 1 mM) was added to an appropriate volume of extracted soluble or membrane fractions (see above) so that the final protein concentration in each case was 10 mg ml⁻¹. Formate (electron donor) was added to a final concentration of 20 mM, and vials containing the membrane/metal mixtures were incubated overnight at 37 °C. Negative controls were prepared similarly with the addition of Tris/HCl buffer (pH 7.5) in lieu of soluble or membrane fraction. The reduction of Pd(II) to Pd(0) was assessed visually through the formation of the characteristic black precipitate, and selected samples were assayed for residual Pd(II) by the SnCl₂ method as above following the removal of the black precipitate by centrifugation.

Preparation of palladium biocatalysts (bioPd(0)). For preparation of bioPd(0) (palladized biomass) of each strain, a known volume of resting cell suspension (see above) was transferred anaerobically into 100 ml serum bottles and an appropriate volume of degassed 2 mM Pd(II) solution (from Na₂PdCl₄ to pH 2.3 with 0.01 M HNO₃) was added so that the final ratio (weight of Pd:dry weight of cells) was 1:19 (5% Pd on biomass for comparison with commercial 5% Pd on carbon catalyst). The cell/Pd mixture was left to stand (30 min, 30 °C) and H₂ was then sparged through the suspension for 10 min to reduce cell-surface-bound Pd(II) to Pd(0). Complete removal of Pd(II) from solution was confirmed by assaying the cell/Pd mixture supernatant for residual Pd(II) by the SnCl₂ method as above. The Pd(0)-coated biomass was harvested by centrifugation (3000 g, 10 min, 25 °C), and washed three times in distilled water and once in acetone. Washed bio-Pd(0) was then resuspended in a small volume (5 ml) of acetone, dried in air to constant weight and finely ground. Chemically produced Pd(0) ('chemPd(0)') was prepared in parallel without the addition of cells. This required 45 min under H₂ for complete reduction to Pd(0) in the absence of biomass. Commercial Pd(0) catalyst (5% Pd on carbon) was a gift from Professor J. M. Winterbottom (Department of Chemical Engineering, University of Birmingham, UK).

Transmission electron microscopy (TEM) and X-ray powder diffraction (XRD) analysis of Pd-loaded biomass.

Pellets of Pd-loaded bacteria (1:2 weight of Pd:dry weight of cells) were rinsed twice with distilled water, fixed in 2.5% (w/v) glutaraldehyde, centrifuged, resuspended in 1.5 ml 0.1 M sodium cacodylate buffer (pH 7) and stained in 1% osmium tetroxide in 0.1 M phosphate buffer pH 7 (60 min) for TEM. 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 at 60 °C). Sections (100–150 nm thick) were cut from the resin block, placed onto a copper grid and viewed with a JEOL 1200CX2 transmission electron microscope; accelerating voltage 80 kV.

For XRD analysis, samples of Pd(II)-challenged biomass and chemically reduced Pd(II) were washed three times in distilled water and once in acetone, resuspended in a small volume of acetone, air-dried and ground to a fine powder in an agate mortar. XRD patterns were acquired as described by Creamer *et al.* (2006) with a Siemens D5005 diffractometer using monochromatic high-intensity CuKα

radiation ($\lambda=0.1240598$ nm). The XRD pattern was compared to references in the Joint Committee for Powder Diffraction Studies (JCPDS) database.

Measurement of the catalytic activity of bioPd(0) via reduction of Cr(VI). The catalytic activity of dried, ground bioPd(0) made from each strain was estimated by the reduction of Cr(VI) (0.5 mM Na_2CrO_4 solution in 20 mM MOPS/NaOH buffer, pH 7.0) to Cr(III). An appropriate amount of catalyst was weighed in 10 ml serum bottles so that each reactor contained 0.5 mg Pd (i.e. 10 mg of 5% bioPd(0)). The catalysts were suspended in 9 ml Cr(VI) solution, and the mixtures were degassed (10 min) and left to equilibrate under OFN (30 min) on a shaker. The reaction was initiated by the addition of sodium formate (1 ml, 25 mM final concentration) and a sample was withdrawn immediately for estimation of the initial Cr(VI) concentration. Timed samples were successively withdrawn, centrifuged (12 000 g, 4 min; IEC Centra M bench centrifuge) and supernatants were assayed by the diphenyl carbazide method (Mabbett *et al.*, 2002) to estimate the residual Cr(VI) concentration. Removal of Cr(VI) was expressed as the percentage of Cr(VI) removed from the solution as compared to the initial concentration.

RESULTS

Involvement of *E. coli* uptake hydrogenases in the bioreduction of Pd(II)

Initial experiments were designed to determine which of the hydrogenase isoenzymes could be involved in bioreduction of Pd(II) by *E. coli*. First, it was necessary to incorporate a 'biosorption step', involving the pre-incubation of cells with Na_2PdCl_4 , in order to obtain bioPd(0) preparations with maximal catalytic activity (Yong *et al.*, 2002a). During the pre-incubation period all suspensions became yellow (including cells deficient in all the major hydrogenases), indicating the adsorption of the $[\text{PdCl}_4]^{2-}$ complex on the cell surface. None of the *E. coli* strains tested (Table 1) displayed impaired ability to biosorb $[\text{PdCl}_4]^{2-}$, confirmed by assay of residual Pd(II) (Fig. 1). This suggests that no active role is played by hydrogenases in the preliminary biosorption step, which was confirmed by the fact that heat-killed cells adsorbed a similar amount of Pd(II) from solution on to their surfaces as the live parental strain, MC4100.

Following the biosorption step the bioreduction of Pd(II) was initiated by the addition of sodium formate to the Na_2PdCl_4 /cell mixtures. Sodium formate was used for these tests because its concentration was accurately known and complete mixing was achieved rapidly. The rate of Pd(II) reduction in each reactor was determined by estimating the amount of residual free Pd(II) ions over 4 h (Fig. 1). Control experiments revealed that the chemical reduction of Pd(II) by formate proceeded slowly, ~ 1.25 mmol Pd(II) being reduced over the time-course of the experiment, with an initial reduction rate of 0.017 $\mu\text{mol Pd(II) reduced min}^{-1}$. These results were not significantly different from those observed for heat-killed *E. coli* MC4100 cells, which had an initial rate of 0.021 $\mu\text{mol Pd(II) reduced min}^{-1}$ and a final amount of Pd(II) reduced of 1.5 mmol (*t*-test, $P>5\%$). In contrast, the formate-dependent reduction of Pd(II) from mixtures including live cells showed increased rates of

Fig. 1. Reduction of Pd(II) from Na_2PdCl_4 by wild-type and hydrogenase-deficient strains of *E. coli* using sodium formate as electron donor. □, *E. coli* MC4100 (parent); ◇, IC009 (Hyd-1 only); △, IC010 (Hyd-2 only); ○, FTD89 (Hyd-3 only); ▲, FTD150 (deficient in all three major hydrogenases). The rates of Pd(II) reduction using heat-killed cells (■) and sodium formate (●) were used for comparison. Data are means $\pm$ SEM from three experiments. Where no error bars are shown, these were within the dimensions of the symbols.

reduction. *E. coli* MC4100, IC009 ($\Delta\text{hyaB } \Delta\text{hycE}$; Hyd-1 only), IC010 ($\Delta\text{hyaB } \Delta\text{hycE}$; Hyd-2 only), and FTD89 ($\Delta\text{hyaB } \Delta\text{hyaC}$; Hyd-3 only) showed initial reduction rates of 0.225 , 0.210 , 0.220 and 0.125 $\mu\text{mol Pd(II) reduced min}^{-1}$ respectively. There was no significant difference ($P=0.15$) between the Pd(II) reduction rates of the parent strain MC4100 and the two mutants that possess a periplasmic-facing uptake hydrogenase (IC009 and IC010). FTD89, which possesses only the cytoplasmic-facing, hydrogen-evolving hydrogenase (Hyd-3), showed significantly slower Pd(II) reduction and the reaction did not go to completion, with ~ 0.2 mM Pd(II) still remaining 4 h after the addition of sodium formate. It was assumed (but not tested) that the slower rate was attributable to the permeability barrier to uptake of Pd(II) through the cytoplasmic membrane and that the residual ~ 0.2 mM Pd(II) represented the limit of affinity of a putative transporter for Pd(II). The mutant strain devoid of all genes encoding [NiFe] hydrogenases (FTD150) had lost the ability to reduce significantly Pd(II) ions from solution, with an initial rate of 0.042 $\mu\text{mol Pd(II) reduced min}^{-1}$. Hydrogenases are therefore key enzymes in the bioreduction of Pd(II) by *E. coli*.

Microscopic analysis of Pd(0) nanoparticles following bioreduction of Pd(II)

In order to assess the subcellular localization of the Pd(0) deposits, aliquots of Pd(II)-challenged cells were examined by TEM (Fig. 2). Cells unchallenged with Pd(II) were indistinct (Fig. 2a) but in Pd(II)-challenged cells, striking differences in the cellular localization of the Pd(0) deposits were observed. Heavy coverage of black Pd(0) deposits was observed around the perimeter of the MC4100 parent strain (Fig. 2b); however, inspection at higher magnification

Fig. 2. TEM of hydrogenase-deficient strains. (a, b) Untreated (a) and palladized (b) cells of MC4100 (parent strain). (c–g) Single cells of Pd(II)-challenged *E. coli* strains demonstrate localization of Pd(0) deposits: (c) MC4100 (parent strain), (d) IC009 (Hyd-1 only), (e) IC010 (Hyd-2 only), (f) FTD89 (Hyd-3 only); (g) FTD150 (lacking all hydrogenases). Cells were loaded using sodium formate as electron donor, harvested by centrifugation and prepared for TEM as described in Methods. Scale bars, 200 nm. Details of membrane-bound Pd(0) deposits are shown in the insets of (e) and (f) (inset scale bars, 50 nm). (h) EDX analysis of bioPd(0) preparations confirms the identity of the black deposits as Pd(0). Cells were loaded with Pd(0) to 50 % of the dry weight in order to visualize the cell-bound deposits.

(Fig. 2c) suggests that the Pd(0) particles are located on both sides of the cytoplasmic membrane. Assuming that the location of the Pd(0) deposits correlates with the location of enzymic Pd(II) reductases, these data suggest that membrane-bound Pd(II) reductases are also present on both sides of the cytoplasmic membrane in the parental *E. coli* strain.

The *E. coli* mutants lacking one or more of the [NiFe] hydrogenases appeared to exhibit different patterns of Pd(0) deposition as compared to the parental strain. Cells of strain IC009 ($\Delta hybC \Delta hycE$; Hyd-1 only) (Fig. 2d) were not heavily covered with extracellular Pd(0) clusters. Indistinct particles were scattered in the periplasmic space, with a small number of particles apparently associated with the outer leaflet (outward facing) of the cytoplasmic membrane (Fig. 2d, arrows). In cells of strain IC010 ($\Delta hyaB \Delta hycE$; Hyd-2 only) (Fig. 2e), little Pd(0) deposition was apparent in the periplasm and a thick outward-facing (Fig. 2e, inset) Pd(0) layer coated the cytoplasmic membrane; a similar membrane-bound pattern of Pd(0) deposition (arrowed) was observed with cells of strain FTD89 ($\Delta hyaB \Delta hybC$; Hyd-3 only) (Fig. 2f). However, in this case, many Pd(0) deposits were facing inwards (Fig. 2f, inset) in accordance with the cytoplasmic-facing location of Hyd-3. The identity of the deposits as Pd(0) was confirmed by EDX analysis (Fig. 2h); the C, O and S components are attributable to cellular material.

These observations, not seen in cells lacking all hydrogenases (Fig. 2g) suggest the presence of multiple membrane-bound Pd(II)-reducing apparatuses in *E. coli*. Although the microscopic evidence presented here is not definitive, since the exact nanoparticle localizations were obscured by the particle overgrowth, these studies of hydrogenase-deficient mutants suggest that the location of the Pd(0) deposits is in accordance with the subcellular localization of the remaining active hydrogenase, as evidenced by the direction of outgrowth shown in Fig. 2(e, f). A similar result was observed previously in mutants of *D. fructosovorans* deficient in their periplasmic hydrogenases, where Pd(0) was localized on the cytoplasmic membrane only (Mikheenko *et al.*, 2008). Microscopic examination of Pd(II)-challenged FTD150 (Fig. 2g), which lacks all major hydrogenases, showed that the Pd(0) deposits associated with this strain were distributed randomly and many were not associated with the cells (Fig. 2g, arrow). Indeed, it is most likely that these Pd(0) deposits did not arise from any biological activity but more probably from chemical Pd(II) reduction, since the clusters were overgrown and did not exhibit the nanoparticulate

format usually observed in bioPd(0) preparations. It is important to note that Pd(0) catalyses the breakdown of formate to H_2 and CO_2 (Mabbett *et al.*, 2006) and hence abiotic generation of H_2 is to be expected, which would explain the ability of the Hyd-1- and Hyd-2-only mutants to reduce Pd(II) at the expense of formate in the absence of active FHL; the strain lacking all major hydrogenases accumulated Pd(0), albeit poorly (Figs 1 and 2). These results indicate that, in addition to being key enzymes involved in Pd(II) reduction, hydrogenases could act as nucleation sites for the growth of Pd(0) nanoparticles. Previous studies used X-ray photoelectron spectroscopy to indicate that amine groups were involved in the mechanism of coordination of Pd(II) from $[PdCl_4]^{2-}$ to the cells (De Vargas *et al.*, 2005).

The XRD patterns of Pd(II)-challenged cells (Fig. 3a) and chemically reduced Pd(II) (Fig. 3b) were compared to the reference database for metallic Pd. This analysis confirmed the cell-mediated reduction of Pd(II) to metallic Pd(0). No additional peaks except those attributable to Pd(0) were detected. The presence of broad, undefined peaks in the cell preparations (Fig. 3a) suggested the presence of poorly crystalline particles and is a clear indication of the presence of Pd(0) nanoparticles. In contrast, the XRD pattern of chemically reduced Pd(II) showed intense sharp peaks similar to bulk Pd(0). No notable differences in the XRD patterns were observed between preparations from wild-type or mutated strains (not shown).

Involvement of membrane-bound hydrogenases in Pd(II) reduction *in vitro*

The above experiments using live cells suggested that membrane-bound hydrogenases were involved in Pd(II) reduction. Next, isolated membranes and soluble proteins were prepared from the MC4100 parent strain and the hydrogenase-deficient mutant FTD150 and each fraction was assessed for its ability to reduce Pd(II) using formate as the electron donor (Fig. 4). Following a 24 h incubation the soluble fractions showed no signs of Pd(II) reduction (not shown). While very slight activity was observed by a darkening of the membrane fraction of the hydrogenase-deficient strain (FTD150), the membrane fraction of MC4100 turned black, indicative of Pd(0) deposition (Fig. 4d). Estimation of the amount of residual Pd(II) ions from the experiments with membrane fractions confirmed that $>95 (\pm 2.3) \%$ of Pd(II) was removed by the MC4100 membrane fractions while a negligible amount

Fig. 3. Formation of Pd(0) by *E. coli*. XRD patterns of (a) Pd(II)-challenged cells of MC4100 and (b) chemically reduced Pd(II) using sodium formate. XRD confirms the reduction of Pd(II) to Pd(0) through the crystallographic identification of the material present in powder samples. The pattern obtained experimentally matches the pattern of Pd(0) found in the International Centre for Diffraction database (JCPDS 46-1043). No additional lines other than those attributable to pure Pd were detected in bioPd(0) preparations. Identical results were obtained using Pd(0) obtained with H_2 as electron donor.

of Pd(II) was removed in vials containing membrane fractions from FTD150. These *in vitro* data add further weight to the hypothesis that membrane-bound hydrogenases are critically involved in the Pd(II) reduction process in *E. coli*. The mechanism by which the hydrogenase-deficient membranes accumulated a small amount of Pd(0) was not investigated, but in this context it should be noted that an additional, unidentified mechanism was shown in the bioreduction of Au(III) (Deplanche & Macaskie, 2008) that awaits further study.

Role of hydrogenases in the production of highly catalytically active Pd nanoparticles by *E. coli*

Next, the catalytic activity of the bioPd(0) prepared from MC4100, IC009, IC010, FTD89 and FTD150 cells was tested and compared to that of chemPd(0) and a commercial catalyst (5 % Pd on carbon). The assay involved following

Fig. 4. Association of Pd(II) reductase activity with the cell membranes. Membrane fractions of (d) MC4100 and (c) FTD150 (lacking all major hydrogenases) after 24 h challenge with Pd(II) amine salt and 20 mM sodium formate. Controls consisted of (a) membrane fractions of MC4100 with sodium formate and (b) $Pd(NH_3)_4Cl_2 \cdot H_2O$ reduction by sodium formate. The colour change indicates Pd(II) reduction to Pd(0). Membrane from the Hyd-deficient mutant appears very pale grey.

the reduction of Cr(VI) to Cr(III) using Pd(0) as a catalyst (Fig. 5) and is predicted by the standard redox potential for the couples Pd(II)/Pd(0) and Cr(VI)/Cr(III) at +0.951 V and -0.13 V, respectively (Lide, 2000). ChemPd(0) preparations exhibited weak catalytic activity in the assay, reducing $9.1(\pm 3.3)$ % Cr(VI) over 3 h. In contrast, the 5 % Pd/Carbon commercial catalyst was highly effective, with $85.3(\pm 1.3)$ % Cr(VI) reduced in 30 min. The bioPd(0) catalyst derived from MC4100 performed as well as the commercial catalyst, with similar initial conversion rates, while Cr(VI) reduction from the bioPd(0) material associated with cells of the mutant depleted of all major hydrogenases (FTD150) was very slow. Deletion of genes encoding Hyd-2 and Hyd-3 (IC009; Hyd 1-only) did not result in a significant loss of catalytic activity of the resultant bioPd(0), while combining deletions of genes encoding both periplasmic-oriented hydrogenases Hyd-1 and Hyd-2 (FTD89; Hyd 3-only), or Hyd-1 and the cytoplasmically oriented Hyd-3 (IC010; Hyd 2-only), distinctly reduced the quality of the resultant bioinorganic catalyst (Fig. 5). It is concluded that the presence of an active Hyd-1 is essential to manufacture bioPd(0) with maximal catalytic activity in the reduction of Cr(VI).

DISCUSSION

In this study we have demonstrated that *E. coli*, a micro-organism with well-defined physiology and genetics, can reduce Pd(II) ions from acidic solutions (pH 2.4), conditions which mimic Pd(II)-containing industrial wastes. The Pd(II) reduction process initially described in SRB (Lloyd *et al.*, 1998) and detailed by Yong *et al.* (2002a, b) was shown to result from the combination of (i) biosorption of the Pd(II)-aqua complexes onto the biomass carboxyl and amine groups (De Vargas *et al.*, 2005) and (ii)

Fig. 5. Catalytic activity of bioPd(0) obtained from various strains, chemPd(0) and a commercial catalyst (5 % Pd/C) in the reduction of 0.5 mM Na₂CrO₄. During the manufacture of the bioinorganic catalysts, cells of each strain were loaded to a mass ratio of 1 : 19 Pd : biomass (5 % Pd on biomass) using H₂. The residual Cr(VI) concentration was estimated at each time point by the diphenyl carbazide method as described in Methods. Results are expressed as the percentage of Cr(VI) reduced over 4 h. □, *E. coli* MC4100; ◇, IC009 (Hyd-1 only); △, IC010 (Hyd-2 only); ○, FTD 89 (Hyd-3 only); ●, FTD 150 (lacking all major hydrogenases); ▲, ChemPd(0); ■, 5 % Pd/C commercial catalyst. Data are means ± SEM from three experiments. Where no error bars are shown, these were within the dimensions of the symbols.

electron-donor-mediated bioreduction of Pd(II) ions into small Pd(0) clusters, followed by (iii) autocatalytic growth of Pd(0) clusters that eventually protrude on the cell surface. These events are, however, not clearly delineated. In addition, De Vargas *et al.* (2005) observed endogenous reduction of Pt(IV) to Pt(II) and Pt(0) using X-ray photoelectron spectroscopy, but the results obtained for Pd(II) were not unequivocal.

Our study suggests a similar sequence of events for Pd(II) reduction by *E. coli*. Experiments with heat-killed cells suggest that the biosorption step does not involve biological activity and probably results from the chemical affinity of the [PdCl₄]²⁻ complex for protonated groups on the cell surface at acidic pH. The peptidoglycan moiety of the bacterial cell wall consists of a repeated motif of *N*-acetylglucosamine and *N*-acetylmuramic acid disaccharide rich in amine groups available for Pd(II) sorption (Meroueh *et al.*, 2006). De Vargas *et al.* (2004) showed that with *D. desulfuricans*, the biosorption equilibrium of Pd(II) was achieved after 30 min at pH values between 0.1 and 3.0; this exposure time was therefore selected in our study. At similar dry biomass:metal ratios (2 : 1), the biosorption capacity of *E. coli* cells is slightly inferior to *D. desulfuricans*, with respective values of ~27 % and ~36 % Pd(II) lost from solution as reported by Mikheenko (2004).

The contribution of *E. coli* cells to Pd(II) reduction is suggested by the fact that formate-mediated chemical Pd(II) reduction was significantly slower in the absence of

biomass (Fig. 1). Several studies have suggested that hydrogenases are the mediating enzymes in this process. Lloyd *et al.* (1998) reported the loss of Pd(II)-reducing ability in Cu²⁺-treated cells of *D. desulfuricans*. Cu²⁺ acts as a selective inhibitor of soluble periplasmic (but not membrane-bound cytoplasmic-facing) hydrogenases in SRB by displacing the [NiFe] active centre of the enzyme. Furthermore, experiments by Mikheenko (2004) on the purified periplasmic [NiFe] hydrogenase from *D. fructosovorans* showed that this enzyme can remain partially active (32 % of original activity was retained) for up to 1 h at pH 2.2.

The work described here was based on the initial assumption that the mechanism of Pd(II) reduction in *E. coli* would be similar to that previously suggested in SRB (Mikheenko, 2004). Indeed, the metal reductase activity of hydrogenases has been observed previously for Tc(VII) (Lloyd *et al.*, 1997), selenite (Yanke *et al.*, 1995), Cr(VI) (Chardin *et al.*, 2003) and U(VI) (Lovley & Phillips, 1992), while recent reports (Deplanche *et al.*, 2007; Konishi *et al.*, 2006) indicate their potential implication in Au(III) reduction in several organisms. We therefore set out to test directly the roles of *E. coli* [NiFe] hydrogenases in Pd(II) reduction. All three hydrogenases could contribute to Pd(II) reduction *in vivo*. Biphasic Pd(II) reduction rates were observed with strains IC009, IC010 and FTD89 (each possessing one of the three major hydrogenases) in a similar way to the parent strain *E. coli* MC4100. Strain FTD150, lacking any fully functional hydrogenase, displayed a linear Pd(II) reduction rate comparable to heat-killed cells and chemically reduced Pd(II). Hence, hydrogenases appear to be required to initiate the formation of Pd(0) 'seeds', which will further lead to the autocatalytic reduction of Pd(II) as well as formate breakdown via Pd(0). Notably, the rate of Pd(II) reduction decreased when Hyd-1 and Hyd-2 were inactivated, leaving only the cytoplasmic-facing Hyd-3.

Formate-dependent Pd(II) reduction by Hyd-1 and Hyd-2 would likely require a different reaction mechanism to that of Hyd-3. Hyd-3 receives its electrons essentially directly from cytoplasmic FdhF formate dehydrogenase within the FHL complex. In the case of formate-dependent Pd(II) reduction by Hyd-1 and Hyd-2, however, the respiratory formate dehydrogenases (FdnGHI and FdoGHI) would first oxidize formate to CO₂ and pass electrons to the quinone pool before *reversed* electron transport from quinol through to the uptake hydrogenases active sites occurs. This scenario is possible here since under the reaction conditions used the quinone pool would likely become overreduced and thus, in the presence of Pd(II) at the hydrogenase active sites, result in reversed electron flow from quinol through Hyd-1 or Hyd-2 to Pd(II). An alternative chemical explanation would require the uptake and endogenous reduction of Pd(II) ions to Pd(0) clusters, which would generate an H₂ pool (thus replacing Hyd-3 function) by Pd(0)-mediated catalytic splitting of exogenous formate. H₂ thus generated could diffuse to the site of

the periplasmic-facing Hyd-1 and Hyd-2 and be used as an electron source for Pd(II) reduction. A previous study using X-ray photoelectron spectroscopy showed evidence for endogenous reduction of Pt(IV) to Pt(0) in the absence of added electron donor, but endogenous Pd(II) reduction was not shown unequivocally (De Vargas *et al.*, 2005).

Since Hyd-3 receives its electrons directly from an uncoupled formate dehydrogenase enzyme (FdhF) the Hyd-3-dependent reduction of Pd(II) at the cytoplasmic side of the membrane may be a more favourable reaction. However, the lower Pd(II) reduction activity observed with the strain bearing only active Hyd-3 (FTD89) suggests this is not the case. It is possible that the additional requirement for transport of Pd(II) across the cytoplasmic membrane before it could be reduced by Hyd-3 is the rate-limiting factor in this case. The nature of the putative transporter(s) required for Pd(II) uptake is not known and hence speculation is unwarranted, but Pd(II) uptake is implied by the presence of small cytoplasmic Pd(0) deposits (Fig. 2c). The paucity of information on bacteria–Pd(II) interactions suggests that future studies would be worthwhile.

Having established the involvement of hydrogenases in the Pd(II) reduction mechanism of *E. coli*, the second objective of this study was to assess the activity of the bionanocatalyst made from each strain (bioPd(0)) and identify the hydrogenase involved in the formation of the most highly catalytic Pd(0) nanoparticles. Similar studies on *D. fructosovorans* and its [Fe]-only hydrogenase-negative mutant (Mikheenko *et al.*, 2008) showed that deletion of the periplasmic hydrogenases increased the catalytic activity of the resulting bioPd(0) in a Cr(VI) reduction assay (Rousset *et al.*, 2006). In contrast, none of the *E. coli* mutants tested in the present study showed a catalytic activity greater than the parent strain. However, the catalytic activity of bioPd(0) was still dependent on the hydrogenase that mediated Pd(II) reduction. Our results suggest that the presence of at least one hydrogenase is crucial in order to obtain a catalyst with an activity significantly superior to chemically reduced Pd(II), and that Hyd-1 produces the best-quality bioPd(0) (Fig. 5). The low activity of bioPd(0) made from FTD89, which lacks periplasmic Hyd-1 and Hyd-2, is not surprising, since the Pd(0) nanoparticles are buried on the inside of the cytoplasmic membrane, as clearly visible in Fig. 2(f) (inset), probably reducing the available reactive Pd(0) surface. In addition to being more numerous, Pd(0) nanoclusters synthesized by the wild-type strain (*E. coli* MC4100) are located on the cell surface and across the periplasmic space, the former probably resulting from the outgrowth of Pd(0) seeds located in the periplasmic space (Fig. 2b). The catalytic activity of such preparations is comparable to that of a commercial catalyst (5% Pd/C). The difference in catalytic activity of bioPd(0) made from IC009 and IC010 is more surprising since both possess one periplasmically oriented uptake hydrogenase. However, TEM examination of

both Pd(II)-treated strains indicated that the pattern of Pd(0) clusters formed by IC009 is very similar to that of the wild-type (i.e. Pd(0) spans the periplasm), although Pd(0) deposits are less numerous, while the IC010 micrographs are comparable to those obtained with FTD89. Although Hyd-2 activity has been found to be responsible for the majority of the total hydrogenase activity in cells grown (as in this study) with fumarate and glycerol (Sawers *et al.*, 1985), this enzyme is usually present in the membrane in lower amounts than Hyd-1 (Ballantine & Boxer, 1985). It is possible that during Pd(II) reduction, Hyd-1 may thus provide a higher number of Pd(II) nucleation foci in the periplasm and result in the formation of a higher number of smaller Pd(0) particles. The micrographs shown in Fig. 2 were obtained at a relatively late stage of Pd(0) deposition, and TEM examination of the early stages of Pd(II) reduction in those two mutants would be required to confirm this hypothesis. However, this was not carried out, as the small size of the Pd(0) deposits makes their definition under the electron microscope difficult. Alternatively, different specific reaction rates between Hyd-1 and Hyd-2 may influence the quality of nanoclusters produced. Assuming that differences of catalytic activity are due to the size and surface area of the palladium particles, these results demonstrate the importance of specific hydrogenase activity in the formation of catalytically active bioPd(0) nanoparticles by *E. coli*. The hydrogenase-1 isoenzyme, encoded by the *hyaABCDEF* operon, appears to be best suited for the production of good-quality, catalytically active bioPd(0) nanoclusters using the Cr(VI) test.

In conclusion, this study provides insight into the mechanism by which *E. coli* can reduce Pd(II) ions in acidic solutions. We suggest that [NiFe] hydrogenases are responsible for accelerating the initial stages of Pd(II) nucleation and the formation of Pd(0) nanoparticles. Production of biohydrogen for clean energy yields waste *E. coli* biomass which has been successfully palladized and shows catalytic activity (Redwood, 2007). A recent survey of various *E. coli* strains that possess various combinations of hydrogenases showed the highest H₂ production using FTD89 (Hyd-3 only) (Redwood *et al.*, 2008). Hence, although bioPd(0) preparations produced from this strain seem to have decreased catalytic activity compared to the parent strain in the Cr(VI) reduction test (this study), future work will focus on comparing bioPd(0) made using the Hyd-1 strain with that made using FTD89 waste biomass arising from biohydrogen production in industrially relevant reactions. These will include selective hydrogenations as well as a function as electrocatalyst in a proton-exchange membrane fuel cell (PEMFC) to make electricity from hydrogen. It is important commercially that the catalyst is made economically on a waste biomass. A survey of several bionanocatalysts in a wide range of aqueous and solvent-based reactions and in fuel cells is in progress in our laboratory and will be reported in full at a later date.

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