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DOI:

[10.1016/j.apcatb.2013.09.045](https://doi.org/10.1016/j.apcatb.2013.09.045)

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Document Version

Publisher's PDF, also known as Version of record

Citation for published version (Harvard):

Deplanche, K, Bennett, JA, Mikheenko, IP, Omajali, J, Wells, AS, Meadows, RE, Wood, J & Macaskie, L 2014, 'Catalytic activity of biomass-supported Pd nanoparticles : influence of the biological component in catalytic efficacy and potential application in 'green' synthesis of fine chemicals and pharmaceuticals', *Applied Catalysis B: Environmental*, vol. 147, pp. 651-665. <https://doi.org/10.1016/j.apcatb.2013.09.045>

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Catalytic activity of biomass-supported Pd nanoparticles: Influence of the biological component in catalytic efficacy and potential application in 'green' synthesis of fine chemicals and pharmaceuticals[☆]



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ARTICLE INFO

Article history:

Received 27 June 2013

Received in revised form

20 September 2013

Accepted 28 September 2013

Available online 9 October 2013

Keywords:

Catalysis

Cr(VI) reduction

Heck coupling

Hydrogenase

Suzuki reaction

ABSTRACT

Five gram negative and two gram positive bacterial strains known for their heavy metal tolerance or ability to reduce metal ions were coated with Pd(0) nanoparticles (NPs) via reduction of soluble Pd(II) ions under H₂ following an initial uptake of PdCl₄²⁻ without added electron donor ('biosorption'), where the gram negative strains had a 5-fold greater capacity for Pd(II) exceptionally; the possibility of reduction to Pd(0) via an endogenous electron donor was not discounted. The initial rate of subsequent H₂-mediated Pd(II) reduction correlated with the Pd(II) removed during biosorption ($r^2 = 0.9$). TEM showed strain-specific variations of Pd-NPs. At a 1:3 loading of Pd:biomass the cell surfaces of *Escherichia coli* and *Desulfovibrio desulfuricans* showed uniform coverage with small NPs with the other strains showing larger aggregates. NPs made by the gram positive cells appeared larger than their gram negative counterparts. At a loading of 1:19 all were active catalysts in Cr(VI) reduction and in two Heck coupling reactions. BioPd_{E.coli} and bioPd_{D.desulfuricans} and bioPd_{A.oxydans} were consistently the best and worst catalysts respectively. BioPd_{E.coli} was further tested as a process catalyst according to industrial protocols in Heck and Suzuki coupling reactions. Laboratory and industrial tests (coupling of phenyl iodide and ethyl acrylate) gave 75% and 78% conversion to ethyl cinnamate, respectively. The biomaterial catalysed Heck and Suzuki reactions using bromoacetophenone and 4-bromoanisole (Heck) and 4-chloroanisole (Suzuki) but not 3-chlorotoluene. In accordance with known chemical catalysis the catalytic efficacy was related to electron-withdrawing substituents on the phenyl ring, with more than 90% conversion (Suzuki) using 4-bromobenzotrifluoride.

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1. Introduction

Due to their often unequalled catalytic properties, platinum group metals (PGMs) are widely used in many catalytic processes of the petrochemical and chemical manufacturing industries. Since the development of PGM-based catalysts as pollution control

devices for the automotive industry, PGM demand and market price have increased concurrently. At the same time, the use of petrochemicals for 'platform' chemical synthesis is of concern due to the dual effects of dwindling oil supplies and the environmental impact of CO₂. Hence more efficient use of increasingly scarce and expensive materials via 'green chemistry' is pressing.

The development of efficient, cost-effective recycling and green chemistry technologies is a first step towards the conservation and sustainable use of resources. Due to limited global resources and the high market value of PGM, their recovery often takes priority over environmental concerns, for example more than 14 tonnes CO₂ is generated per kilo of Pt produced from primary sources [1]. Traditional PGM recycling methods (electrochemical recovery, solvent extraction) are energy-demanding or rely on the use of aggressive chemicals [2,3].

The reduction of transition metals by some bacteria is believed to exert a considerable impact on the ecology of the environment

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[4]. This microbial ability can be harnessed for biotechnological applications such as bioremediation of metal contaminants and metal biorecovery [5,6]. Recent work on the biorecovery of precious metals (Ag, Au, Pd, Pt) demonstrated the efficacy of this approach from solutions of metal salts as well as from secondary waste sources. In the latter such biorecovered metal can be more effective than pure metal when subsequently used as a catalyst e.g. [7,8], a 'step change' which offers potential clean alternatives to traditional metal refining, greater efficiency with respect to continued supply of rare metals and 'green' synthesis of pharmaceuticals and fine/platform chemicals via catalysts biofabricated from wastes.

Bacterially bound catalytic nanoparticles (NPs) can be comparable to or more effective catalysts than traditional homogeneous or heterogeneous catalysts in hydrogenation [9] and in a classical synthetic reaction, the Heck coupling [10] and they also show high stability for recovery and recycle, attributed to a reduced tendency for the nanoparticles to agglomerate when stabilised by the biomatrix [10].

The continuous recovery of Pd and Pt from spent automotive catalyst leachates was first shown by Yong et al. [11] using an electrobioreactor coated with a biofilm of the sulfate-reducing bacterium (SRB) *Desulfovibrio desulfuricans*. Later work used palladised biofilm of a *Serratia* sp. as a catalyst for continuous detoxification of highly toxic and environmentally problematic Cr(VI) to less toxic Cr(III) in flow-through reactors interrogated via magnetic resonance imaging [12]. Heat-treated palladised cells of *D. desulfuricans* produced an applied film (in activated carbon) electrocatalyst for a fuel cell (FC) anode that produced power comparably to a commercial fuel cell catalyst [13]; use of molecular biology tools later enhanced the corresponding activity of *Escherichia coli*-based FC catalyst [14] that was improved still further by sourcing the metallic catalyst from mixed PGMs from a commercial waste water [15].

Early attempts to elucidate the precise mechanism of Pd deposition and NP synthesis implicated a role for bacterial hydrogenases in Pd(II) reduction [16]. Later, Mikheenko et al. [17] constructed strains of *Desulfovibrio fructosovorans* deficient in its periplasmic hydrogenases, observing relocation of the Pd(0) deposits to the cytoplasmic membrane, the site of the remaining hydrogenase. Similar results were obtained with hydrogenase-deficient *E. coli* mutants [18]. Due to their role in formate and H₂ metabolism the cytoplasmic Hyd-3 is a component of the formate hydrogenlyase (FHL) complex responsible for formate oxidation under fermentative conditions while periplasmic Hyd-1 and Hyd-2 operate as 'uptake' hydrogenases for energy conservation under fermentative (Hyd-1) and anaerobic respiratory (Hyd-2) conditions), and direct evidence [17,18] hydrogenases are implicated in a primary step of Pd(II) bioreduction and to act as a focus for the initial formation of Pd(0) 'seeds', on the hydrogenase itself or on nearby biochemical entities. These 'seeds', in turn, promote further abiotic autocatalytic Pd(II) reduction to Pd(0) [19,20] or of Pt(IV) to make a metal catalyst [8]. This mechanism has been affirmed [21]. Once an initial 'seed' of Pd is formed (regardless of whether via non-enzymatic or enzymatically steered mechanisms) further abiotic autocatalytic metal deposition continues, even from concentrated acids [8,11,22], with the synthesis steered by the initial patterning and also by the nature of the surrounding biochemical ligands [23]. A high resolution TEM study [10] showed unique Pd atomic arrangements at the interface with the supporting biomatrix over and above the Pd NP itself and extending from it but the reactivity of the interface as compared to the bulk NP is not known.

The cell-bound Pd nanoparticles (bioPd) exhibit remarkable catalytic properties in a number of test reactions of environmental and 'green chemistry' significance (see for reviews [7,24,25]). Pd biocatalysts made from various strains, or mutants of a single strain, show variable activity for a given reaction [14,18,26] probably attributable to strain-specific differences in cell surface

composition/patterning and/or the enzymatic apparatus responsible for Pd(II) reduction. Early studies suggested that the pattern of deposition of Pd nanoparticles on the cell surface (and the resulting catalytic activity of the bioPd) is a function of both the initial biosorption of Pd(II) (crystal nucleation) and the subsequent bioreduction to Pd(0) [19,27]. Critically, the ability to 'adjust' the sites of Pd(0) deposition via molecular engineering is a key feature which sets enzymatic nucleation apart from simple biosorption methods.

The gram negative cellular envelope contains many potential Pd(II) nucleation sites, e.g. the peptidoglycan moiety of the bacterial cell wall (located in the periplasm between the cytoplasmic and outer membranes) is rich in carboxyl and amine groups, the preferred ligands for coordination of Pd(II) complexes [28], as well as various proteins, some of which co-extract with Pd nanoparticles [10]. In *D. fructosovorans* two hydrogenases are located in the periplasm (an aqueous gel matrix) and their removal re-locates Pd(0) to the inner membrane site of the remaining hydrogenases (a lipid environment) [17] with corresponding effect on the catalytic activity of the bioPd formed [26].

In contrast to gram negative organisms, gram positive strains have been less well studied with respect to bioPd manufacture although a strain of *Bacillus sphaericus* was highlighted due to its ability to make catalytically active bioPd [9], probably via deposition of Pd(0) onto the protein array of its external S-layer [29]. In *Bacillus* more recent work Lin et al. [30] have suggested a second mechanism whereby Pd(0) locates within the wall layers beneath the S-layer with hydrolysates of polysaccharides or peptidoglycan serving as endogenous electron donor in *B. licheniformis*, in accordance with the observation of Pd(0) layer beneath the S-layer in *B. sphaericus* [31].

Gram negative and gram positive strains present major differences in cell wall composition, the former having a more complex double-membrane structure. The outer membrane assembly (comprising membrane phospholipids with extruded lipopolysaccharide chains) and inner cytoplasmic membrane enclose peptidoglycan within a discrete aqueous gel 'compartment' (the periplasm). Gram positive bacteria have a cell membrane but lack the outer membrane (i.e. they have no periplasm) but they often have an 'S-layer' protein array located beyond a peptidoglycan layer, thicker but of similar composition to that found in the gram negative periplasm. Beveridge [32] describes two types of gram positive bacteria. The first, typified by *B. sphaericus*, have a relatively thin peptidoglycan layer which may be overlaid with S-layer, whereas the second type (e.g. *Arthrobacter*, *Micrococcus*) have thick, robust walls with a thicker peptidoglycan layer. This second type has not been examined previously in the context of deposition of Pd(0).

Spectroscopic studies on Pd(II) reduction on the surface of the S-layer of *B. sphaericus* showed preferred coordination of Pd(II) complexes to carboxyl groups of this protein [29], with non-enzymatic reduction of Pd(II) to Pd(0) to produce bioPd NPs of similar activity as hydrogenation catalyst to the enzymatically made NPs on *D. desulfuricans* [9].

Although the *B. sphaericus* and *D. desulfuricans* exemplars of protein-supported Pd-nanoparticles are now reasonably well understood, future rational catalyst design targeted to specific reactions dictates that the precise microbial mechanisms (and their interplay) that dictate catalyst activity and also specificity are known; the literature will otherwise continue to expand with a potentially infinite catalogue of untargeted, largely descriptive examples.

As a step change towards catalyst targeting for 'green chemistry' applications (i.e. greater selectivity and reaction specificity and reduced waste) in this work gram negative and gram positive strains with known high metal tolerance and/or hydrogenase activity were allowed to deposit Pd nanoparticles under a common set of conditions and the resulting bioPd catalysts were

compared in three independent reactions. The 'reference' material, bioPd_{D. desulfuricans}, was validated previously against homogeneous colloidal Pd catalyst and 5% Pd on carbon, outperforming both in the Heck reaction [10]. The strain with the greatest potential by several criteria was then evaluated commercially via the Heck and Suzuki reactions to establish its utility with a variety of substrates.

Waste bacteria from a primary fermentation (hydrogen producing reactions: a source of clean H₂ for fuel cells [33]) can be readily converted into bioPd catalyst (H₂-consuming reactions) via the reversible hydrogenase activities; bioPd made using such recycled bacteria is catalytically active [14,34] and a promising outcome from the pilot study we report here would warrant a concerted program for dual conversion of waste bacteria and waste metals into a new generation of 'catalysts from wastes' into competitive bioproducts for fine chemistry and pharmaceutical applications.

2. Materials and methods

2.1. Microbial strains and culture conditions

The following were used in this study: gram negative strains *D. desulfuricans* NCIMB 8307, *E. coli* MC4100, *Serratia* sp. NCIMB 40259, *Shewanella oneidensis* MR1 NCIMB 146063 and *Cupriavidus metallidurans* NCIMB 14178. Gram positive strains were *Arthrobacter oxydans* NCIMB 10504 and *Micrococcus luteus* NCIMB 9278. The major characteristics of each strain are shown in Table 1 alongside growth conditions and those used for palladisation experiments.

For bioPd manufacture, cells were grown in 2 l of medium inoculated using an overnight preculture (10% (v/v)) grown in the same way (Table 1). Mid-logarithmic phase cultures (OD₆₀₀ = 0.5–0.7) were harvested by centrifugation (12,000 × g, 15 min), washed three times in 100 ml of MOPS-NaOH buffer (20 mM, pH 7.2), resuspended in 50 ml of the same buffer and stored at 4 °C as concentrated cell suspensions until use, usually the next day. Cell concentration (mg ml⁻¹) was determined by correlation to a pre-determined OD₆₀₀ to dry weight conversion.

2.2. Pd(II) solution

Pd(II) solution (2 mM, pH 2.3) was made by dissolving an appropriate amount of sodium tetrachloropalladate (Na₂PdCl₄, Sigma-Aldrich, Poole, U.K) in 0.01 M HNO₃.

2.3. Preparation of bioPd(0) catalysts

Each strain was palladised as follows. A known volume of the concentrated resting cell suspension was transferred under appropriate oxygen (Table 1) conditions into 200 ml serum bottles and an appropriate volume of degassed 2 mM Pd(II) solution was added so that the final ratio (weight of Pd:dry weight of cells) was 1:19 to give a final loading of 5% Pd on biomass, or as otherwise stated (final pH 2.3). Cells/Pd mixtures were left to stand (30 min, 30 °C) with occasional shaking to promote biosorption of Pd(II) complexes [19] before H₂ was sparged through the suspension for 10 min or as described (0.2 atm) to reduce cell surface-bound Pd(II) to Pd(0). The Pd(0)-coated biomass was harvested by centrifugation (3000 × g,

10 min, 25 °C) and washed three times in distilled water (dH₂O). Following a final acetone wash, the black precipitate was left to dry in air overnight. Black bioPd powder was finely ground in a mortar and tested for catalytic activity without any further processing.

2.4. Assay of Pd(II)

Following the addition of cells to the Pd(II) solution, the concentration of residual Pd(II) ions in solution (sample supernatants) was monitored at all stages of bioPd preparation using the spectrophotometric method of Charlot [35] using timed samples (1 ml) withdrawn and separated (12,000 × g, 4 min, IEC Centra M bench centrifuge). The Sn(II) reagent was made by dissolving 29.9 g of SnCl₂ powder into 500 ml of concentrated HCl. For Pd(II) assay, 200 μl of sample was added to 800 μl of SnCl₂ solution (1.5 ml plastic cuvette) and A₄₆₃ was determined after 1 h (30 °C) against a blank prepared in the same way. The system obeyed Beer's law over the range 5–80 ppm Pd(II). The assay method was validated by analysis of reference and selected test samples by a commercial laboratory (H2b, Capenhurst, U.K.).

2.5. Transmission electron microscopy (TEM) of Pd-loaded bacteria

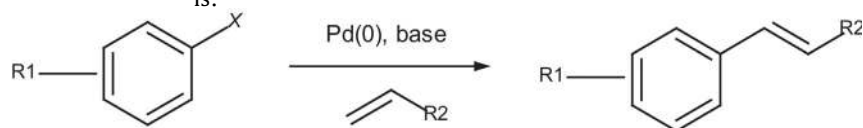
Pellets of Pd-loaded bacteria (25%, w/w Pd on biomass) were rinsed twice with distilled water, fixed in 2.5% (w/vol) glutaraldehyde, centrifuged, resuspended in 1.5 ml of 0.1 M cacodylate buffer (pH 7) and stained in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7 (60 min) for transmission electron microscopy (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 polymerise (24 h; 60 °C). Sections (90–100 nm thick) were cut from the resin block, placed onto a copper grid and viewed with a JEOL 1200CX2 TEM; accelerating voltage 80 kV.

2.6. Evaluation of catalytic activity in aqueous solution: reduction of Cr(VI)

BioPd (10 mg of 5% preparation i.e. 0.5 mg Pd) catalyst was accurately weighed (in triplicate) in 12 ml serum bottles sealed with butyl rubber stoppers. Cr(VI) test solution (5 ml of a 500 μM Cr(VI) solution (Na₂CrO₄·4H₂O) in 20 mM MOPS-NaOH buffer pH 7.0) was added and the mixture was made anaerobic by degassing (10 min) and sparging with oxygen free nitrogen (OFN, 10 min). Bottles were pre-equilibrated (10 min) on a rotary shaker before the reaction was initiated by adding 1 ml of sodium formate (electron donor, final concentration 25 mM). Samples were periodically withdrawn via rubber septa, centrifuged (4 min, 13000 rpm to separate the catalyst) and the supernatant was analysed for residual Cr(VI) using diphenyl-carbazide (DPC) [36]. Reduction of Cr(VI) was estimated as % Cr(VI) reduced at set time points during the experiment (typically 3 h).

2.7. Evaluation of catalytic activity via Heck coupling in solvent: initial laboratory tests

A vital C–C bond forming reaction in organic chemistry is the coupling of an aryl halide or pseudo-halide with an alkene to give an aryl alkene, known as a Heck reaction. The overall reaction scheme is:



Heck couplings of phenyl iodide with ethyl acrylate or styrene over bioPd catalysts were performed as follows: to a 50 ml 2-neck round bottomed flask was added the solvent (dimethylformamide, 15 ml), phenyl iodide (PhI; 1 mmol, 0.204 g), triethylamine

Table 1
Strains and growth conditions used in this study.

Strain	Gram	Respiratory mode	Growth conditions	Rationale	References
<i>Arthrobacter oxydans</i>	+	Strict aerobe	TSB, 25 °C, aerobic ^a	Metal resistant, accumulates metal	[54-56,60]
<i>Micrococcus luteus</i>	+	Strict aerobe	NB N°2, 30 °C, aerobic ^a	Accumulates high amounts of metals, metal-reducer	[50,53,59]
<i>Desulfovibrio desulfuricans</i>	-	Strict anaerobe	Pos C, 30 °C, anaerobic ^b	Metal reducer, high hydrogenase activity	[4,16,104-107]
<i>Escherichia coli</i>	-	Facultative anaerobe	NB N°2, 37 °C, anaerobic respiratory ^c	Metal-reducer, hydrogenase activity	[18,93,94,108]
<i>Serratia</i> sp.	-	Facultative anaerobe	NB N°2, 30 °C, aerobic ^a	Accumulates metals	[109]
<i>Shewanella oneidensis</i>	-	Facultative anaerobe	TSB, 30 °C, anaerobic respiratory ^c	Metal-reducer, hydrogenase activity	[51,52,110]
<i>Cupriavidis metallidurans</i>	-	Facultative anaerobe	TSB, 30 °C, anaerobic ^b	High metal resistance, O ₂ -tolerant hydrogenase	[57] and ref therein

TSB: Tryptone Soya Broth (Oxoid Ltd., Basingstoke Hampshire, UK).

NB N°2: Nutrient Broth N°2 (Oxoid Ltd., Basingstoke Hampshire, UK).

Pos C: Postgate C medium prepared as described in [16].

^a 21 flasks filled with 1 l medium, shaking at 120 rpm.

^b 21 Durham bottles filled almost to the brim with medium, degassed (30 min) and sparged with oxygen-free nitrogen (30 min).

^c 21 Durham bottles filled almost to the brim with medium supplemented with fumarate (0.4%, w/v) and glycerol (0.5%, v/v), degassed (30 min) and sparged with oxygen-free nitrogen (30 min).

(1.5 mmol, 0.2 ml) and catalyst (10.6 mg of 5% bioPd, 0.5 mol% Pd relative to halide) under a nitrogen atmosphere. The mixture was heated to 120 °C with rapid stirring and allowed to equilibrate. The reaction was initiated by the addition of the alkene (ethyl acrylate or styrene, 1.5 mmol, 0.16 and 0.17 ml respectively) and allowed to proceed for 2 (ethyl acrylate) or 4 h (styrene). Timed samples were withdrawn and the reaction products were analysed by HPLC on an analytical Luna 10u C18 column (Phenomenex, Utrecht, NL) using a water-acetonitrile gradient and absorbance detector (230 nm).

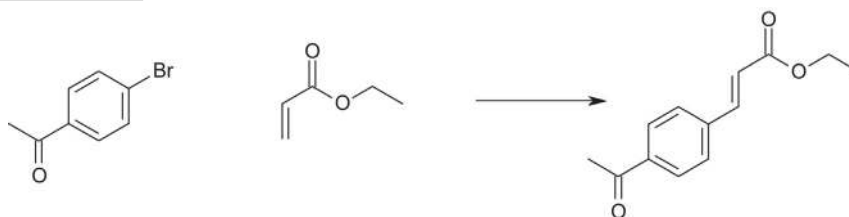
2.8. Further evaluation of catalytic activity: Heck and Suzuki couplings tested by AstraZeneca

For further testing Pd(0) was loaded onto cells of the best strain (*E. coli*) at 2.5% mass (w/w); this metal thrifting had no effect on the

bioPd_{*E. coli*} (38 mg, 0.009 mmol Pd-0.3 mol% Pd loading). The reaction mixture was heated to 110–130 °C for 2 h. After cooling to ambient temperature, water (30 ml) was added and the product was extracted with ethyl acetate (2 × 30 ml), washed with saturated brine (30 ml), dried over MgSO₄, filtered and the solvent removed under vacuum. Crude product was then purified by flash chromatography (20:1 iso-hexane/ethyl acetate) to give the ethyl cinnamate (411 mg, 2.33 mmol, 78% yield).

¹H NMR (500 MHz, CDCl₃) $d_H = 7.69$ (d, $J = 16.0$ Hz, 1H, =CH), 7.54–7.52 (m, 2H, 2x Ar-H), 7.39–7.38 (m, 3H, 3x Ar-H), 6.44 (d, $J = 16.0$ Hz, 1H, =CH), 4.27 (q, $J = 7.2$ Hz, 2H, CH₂), 1.34 (t, $J = 7.1$ Hz, 3H, CH₃) ppm. ¹³C NMR (100 MHz, CDCl₃) $d_C = 167.0$ (C=O), 144.6 (=CH), 134.5 (C), 130.5 (CH), 130.2 (2x CH), 128.1 (2x CH), 118.3 (=CH), 60.5 (CH₂), 14.3 (CH₃) ppm.

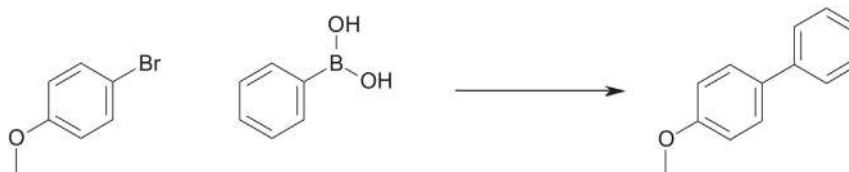
2.8.2. Ethyl (E)-3-(4-acetylphenyl)prop-2-enoate



The reaction was carried out according to general procedure for Heck reaction 2 (Section 2.8.1) using 4-bromoacetophenone (597 mg, 3.0 mmol) at 120–130 °C. The reaction was complete after 5 h. Crude product was purified by flash chromatography (9:1 iso-hexane/ethyl acetate) to give ethyl (E)-3-(4-acetylphenyl)prop-2-enoate (348 mg, 1.59 mmol, 54% yield).

¹H NMR (400 MHz, CDCl₃) $d_H = 7.97$ (d, $J = 8.5$ Hz, 2H, 2x Ar-H), 7.69 (d, $J = 16.0$ Hz, 1H, =CH), 7.60 (d, $J = 8.4$ Hz, 2H, 2x Ar-H), 6.51 (d, $J = 16.0$ Hz, 1H, =CH), 4.28 (q, $J = 7.1$ Hz, 2H, CH₂CH₃), 2.61 (s, 3H, C(O)CH₃), 1.35 (t, $J = 7.1$ Hz, 3H, CH₂CH₃) ppm. ¹³C NMR (400 MHz, CDCl₃) $d_C = 195.5$ (C=O), 164.7 (C=O), 141.2 (=CH), 137.1 (C), 136.3 (C), 127.1 (2x CH), 126.4 (2x CH), 119.1 (=CH), 59.0 (CH₂), 24.9 (CH₃), 12.6 (CH₃) ppm.

2.8.3. General procedure for Suzuki reaction (4-methoxybiphenyl)



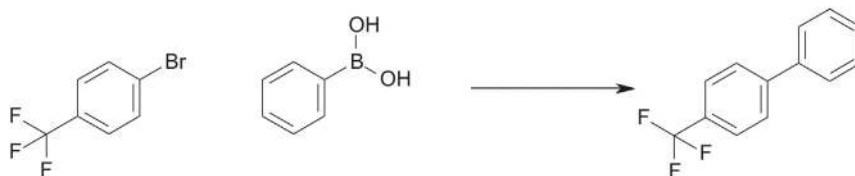
catalytic activity of the Pd(0) produced [8]. Reactions were taken to completion to establish the conversion efficiency (or as otherwise noted), established by monitoring via TLC. Products were extracted as described and the yield was determined gravimetrically (mass) against the mass of starting material (not including any sublimation, which was not measured) and products were confirmed using ¹H and ¹³C NMR.

2.8.1. General procedure for Heck reaction 2 (ethyl cinnamate)

A round bottomed flask was charged with sodium carbonate (318 mg, 3.0 mmol) and N-methylpyrrolidinone (15 ml) was added. To this was added phenyl iodide (335 μ l, 3.0 mmol), ethyl acrylate (326 μ l, 3.0 mmol), triethylamine (418 μ l, 3.0 mmol) and 2.5%

A round bottomed flask was charged with phenyl boronic acid (439 mg, 3.6 mmol), sodium carbonate (636 mg, 6.0 mmol), 2.5% bioPd_{E. coli} (2.8 mg, 0.003 mmol-0.1% Pd loading) and John-Phos ligand ((2-biphenyl)di-tert-butylphosphine; 10.5 mg, 0.03 mmol). To this was added ethanol (9 ml), water (3 ml) and 4-bromoacetophenone (597 mg, 3.0 mmol) and the mixture was heated to 80 °C. The reaction was complete after 18 h. Water (10 ml) was added and the product extracted with ethyl acetate (3 × 10 ml), washed with brine (10 ml), dried over MgSO₄, filtered and the solvent removed under vacuum. Crude product was then purified by flash chromatography (undiluted iso-hexane) to give the 4-methoxybiphenyl as a colourless crystalline solid (343 mg, 1.86 mmol, 62% yield).

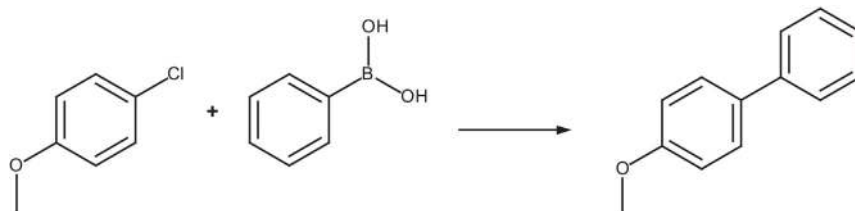
2.8.4. 4-Trifluoromethylbiphenyl



The reaction was carried out according to general procedure for Suzuki reaction using: 4-bromobenzotrifluoride (421 μl, 3.0 mmol). Flash chromatography (neat iso-hexane) gave the product as a colourless crystalline solid (602 mg, 2.71 mmol, 90% yield).

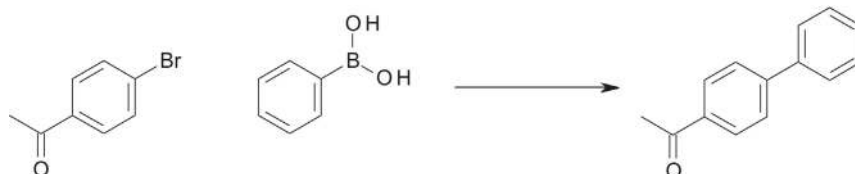
¹H NMR (400 MHz, CDCl₃) *d*_H = 7.69 (s, 4H, 4x Ar-H), 7.61–7.58 (m, 2H, 2x Ar-H), 7.49–7.45 (m, 2H, 2x Ar-H), 7.42–7.38 (m, 1H, Ar-H) ppm. ¹³C NMR (100 MHz, CDCl₃) *d*_C = 144.7 (C), 139.8 (C), 129.4 (q, *J* = 32 Hz, CF₃), 129.0 (2x CH), 128.2 (CH), 127.4 (2x CH), 127.3 (2x CH), 125.7 (q, *J* = 3.8 Hz, 2x CH) ppm.

2.8.5. 4-Methoxybiphenyl



The reaction was carried out according to general procedure for Suzuki reaction using 4-chloroanisole (364 μl, 3.0 mmol) in reaction 5 and p-bromoanisole (378 μl, 3.0 mmol) in reaction 6. Crude product purified by flash chromatography (undiluted iso-hexane) to give the product as a colourless crystalline solid (74 mg, 0.4 mmol, 17% yield). NMR as above.

2.8.6. 4-Acetylbiphenyl



The reaction was carried out as above using: 4-bromoacetophenone (597 mg, 3.0 mmol), heated to reflux overnight. Some product was observed by TLC. 23% product found by ¹H NMR (71% starting material, 6% by-product).

¹H NMR (500 MHz, CDCl₃) *d*_H = 8.03 (d, *J* = 8.5 Hz, 2H, 2x Ar-H), 7.69 (d, *J* = 8.5 Hz, 2H, 2x Ar-H), 7.63 (dd, *J*₁ = 8.5 Hz, *J*₂ = 1.4 Hz, 2H, Ph-H_o), 7.47 (app. t, *J* = 7.3 Hz, 2H, Ph-H_m), 7.40 (tt, *J*₁ = 7.4 Hz, *J*₂ = 1.2 Hz, 1H, Ph-H_p), 2.64 (s, 3H, CH₃) ppm.

3. Results

3.1. Bioaccumulation of Pd(II) in the absence of exogenous electron donor

Previous studies have suggested that a biosorption step at acidic pH is required for bioPd preparations with maximum catalytic activity [19,20]. de Vargas et al. [37] showed that, in the absence of external electron donor, the cell/Pd(II) biosorption equilibrium is typically reached after 30 min. No attempt was made in this study to delineate biosorption (complex formation between soluble metallic ions onto biomass surface ligands) and any reduction of metal via endogenous electron donor. A previous study (using *Desulfovibrio* [28]) used X-ray photoelectron spectroscopy to show some endogenous reduction of Pt(IV) to Pt(II) and Pt(0); clear results could not

be obtained for Pd(II). The concentration of soluble Pd(II) was estimated immediately after the addition of cells and then following a 30 min incubation period to determine the exogenous electron donor-independent ('passive') Pd(II) accumulation capacity.

The Pd(II) removed from the cell/Na₂PdCl₄ mixtures without exogenous electron donor is shown in Fig. 1. The passive Pd(II) removal varied from 4.8 to 121.2 μmol Pd(II) removed g cells⁻¹ for *M. luteus* and *C. metallidurans* respectively; the overall ranking was *M. luteus* < *A. oxydans* < *Serratia* sp. < *S. oneidensis* < *D. desulfuricans* < *E. coli* < *C. metallidurans*. There were marked differences

between the passive Pd(II) capacity of gram positive and gram negative strains. The average Pd(II) accumulation capacity of the latter without added electron donor (111.9 ± 6.7 μmol Pd(II) g cell dry weight⁻¹) was 7 times higher than that of the gram positive strains. Gram negative strains were pre-grown anaerobically (except in the case of *Serratia* sp., see Table 1 and later discussion) while the gram positive strains, being strict aerobes, were grown with O₂. However, preliminary experiments using aero-

bically grown cells of *E. coli* showed similar levels of passive Pd(II) accumulation to their anaerobically grown counterparts (not shown) while catalysis by bioPd made with aerobically pregrown *Serratia* sp. (i.e. non-hydrogenase-mediated Pd(II) reduction in a facultatively anaerobic enterobacterium) was described in detail by Beauregard et al. [12] and Macaskie et al. [38] and by Foulkes et al. [39] with *E. coli* but has not been formally compared with cells pre-grown anaerobically.

Table 2
Amount of Pd(II) removed at various stages of bioPd preparation.

Strain	% Pd(II) removed ^a after				
	Biosorption	1 min H ₂	5 min H ₂	10 min H ₂	Initial Pd(II) reduction rate ^b
<i>A. oxydans</i>	10.3	67.1	98.5	>99	21.9
<i>M. luteus</i>	20.8	77.2	>99	>99	10.9
<i>E. coli</i>	27.2	67.9	>99	>99	32.9
<i>D. desulfuricans</i>	52.7	>99	>99	>99	38.3
<i>Serratia</i> sp.	51.6	95.1	>99	>99	32.3
<i>S. oneidensis</i>	73.8	78.4	>99	>99	31.2
<i>C. metallidurans</i>	98.5	>99	>99	>99	NC ^c
Cell-free	NA	1.3	8.6	19.4	NA

NA: not available.

^a Pd(II) is expressed as % of initial Pd(II) concentration estimated immediately after the addition of cells to the Pd(II) solution. Data are means ± SEM from two separate experiments.

^b Initial Pd(II) reduction rate expressed in μmoles Pd(II) reduced/min/g cells over 0–2 min.

^c NC: not calculated as the majority of soluble Pd(II) ions were removed before addition of electron donor.

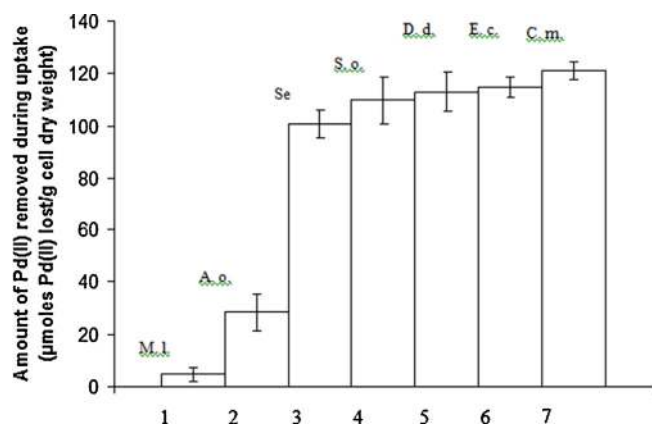


Fig. 1. Electron donor-independent uptake of Pd(II). From left to right on the bar chart: (1) *M. luteus*, (2) *A. oxydans*, (3) *Serratia* sp., (4) *S. oneidensis*, (5) *D. desulfuricans*, (6) *E. coli* and (7) *C. metallidurans*. The amount of Pd(II) in the supernatant was quantified immediately after the addition of cells to the PdCl₄²⁻ solution and after 30 min incubation at 30 °C with occasional shaking. Pd(II) uptake over this biosorption period is expressed as μmoles of Pd(II) removed/g of dry weight of cells. Data are means ± SEM from two separate experiments.

3.2. Reduction of Pd(II) under H₂

Following exogenous electron donor-independent uptake of Pd(II) (above), H₂ gas was sparged into the cell/Pd(II) mixtures and the amount of residual Pd(II) was monitored (Table 2). With all strains, a black Pd(0) precipitate was apparent within 1 min of H₂ addition and after 5 min the concentration of free Pd(II) ions in all cell/Pd(II) mixtures was insignificant. Pd(II) bioreduction was more rapid than chemical Pd(II) reduction under H₂, the latter requiring 45 min under H₂.

The initial Pd(II) reduction rates (μmol Pd(II) reduced min⁻¹ g cells⁻¹) were calculated (Table 2), being the most rapid with *D. desulfuricans*, with initial rates using *E. coli*, *Serratia* sp. and *S. oneidensis* being similar, and 16% less respectively (Table 2). The initial rate using *C. metallidurans* was not calculated because, in this case, the majority of free Pd(II) ions were removed during the initial uptake step. However, an observed instantaneous formation of a black precipitate upon H₂-sparging confirmed rapid Pd(II) reduction. *M. luteus* and *A. oxydans* showed significantly lower initial Pd(II) reduction rates which may be related to the low Pd(II)-uptake capacity of these two strains (i.e. formation of limited nucleation foci). A comparison of Pd(II) biosorption capacity against Pd(II) bioreduction rate confirmed the link between those two parameters correlation coefficient (r^2) was 0.90 (Table 3).

Table 3
Correlation of various parameters of bioPd manufacture with catalytic activity in test reactions.

Correlated to	r^2	
Pd(II) initial uptake	Pd(II) bioreduction rate	0.9043 ^a
	Cr(VI) reduction	0.0365
	Heck coupling of PhI and styrene	0.1485
	Heck coupling of PhI and ethylacrylate	0.4258
Pd(II) bioreduction rate	Cr(VI) reduction	0.1474
	Heck coupling of PhI and styrene	0.0691
	Heck coupling of PhI and ethylacrylate	0.3492
Cr(VI) reduction	Heck coupling of PhI and styrene	0.0318
	Heck coupling of PhI and ethylacrylate	0.1246
Heck coupling of PhI and styrene	Heck coupling of PhI and ethylacrylate	0.7122

^a Correlation is between bioreduction rate (μmoles Pd(II) reduced/min/g cell dry weight) following biosorption and the initial biosorptive uptake of Pd(II) (μmoles Pd(II) removed/g cell dry weight). Pooled data for the 7 strains.

3.3. TEM examination of Pd loaded cells

At a low Pd loading (1:19 mass of Pd:dry weight of cells; 5% by mass), the visualisation of individual Pd nanoparticles (NPs) on the surface of bioPd catalysts is difficult. Therefore, for each strain, cells were loaded in parallel at 25% Pd (w/w) for TEM analysis. Pd(II) reduction was monitored throughout sample preparation as before to confirm that the increase in Pd loading did not lead to changes in the pattern of biosorption/bioreduction.

Fig. 2 shows, for each strain, comparative electron micrographs of native and palladised cells. Pd appeared as electron opaque deposits (identified previously as Pd by energy dispersive X-ray analysis [16]) around the cell periphery. Although the mass ratio of Pd:dry weight of cells was held constant at 1:3, differences in the size and distribution of the Pd NPs were apparent. The majority of *E. coli* and *D. desulfuricans* cells (Fig. 2A and B) were fully surrounded with small NPs while all other strains showed a more random pattern of larger (up to 50 nm) Pd(0) deposits, i.e. some cells were fully surrounded by small Pd clusters while others only possessed a limited number of larger ones. It must be stressed that these differences in Pd patterning are not likely to be attributable to variations in metabolic activity between strains since special care was taken to harvest cells consistently in the mid-logarithmic phase. The Pd deposits on the two gram positive strains (Fig. 2F and G) appeared consistently larger overall than those of the four gram negative strains, especially in the case of *A. oxydans* which apparently did not precipitate Pd(0) as nanoparticles (Fig. 2G).

The localisation of Pd clusters in all but one strain (*A. oxydans*) was restricted to the cell surface and to the periplasmic space

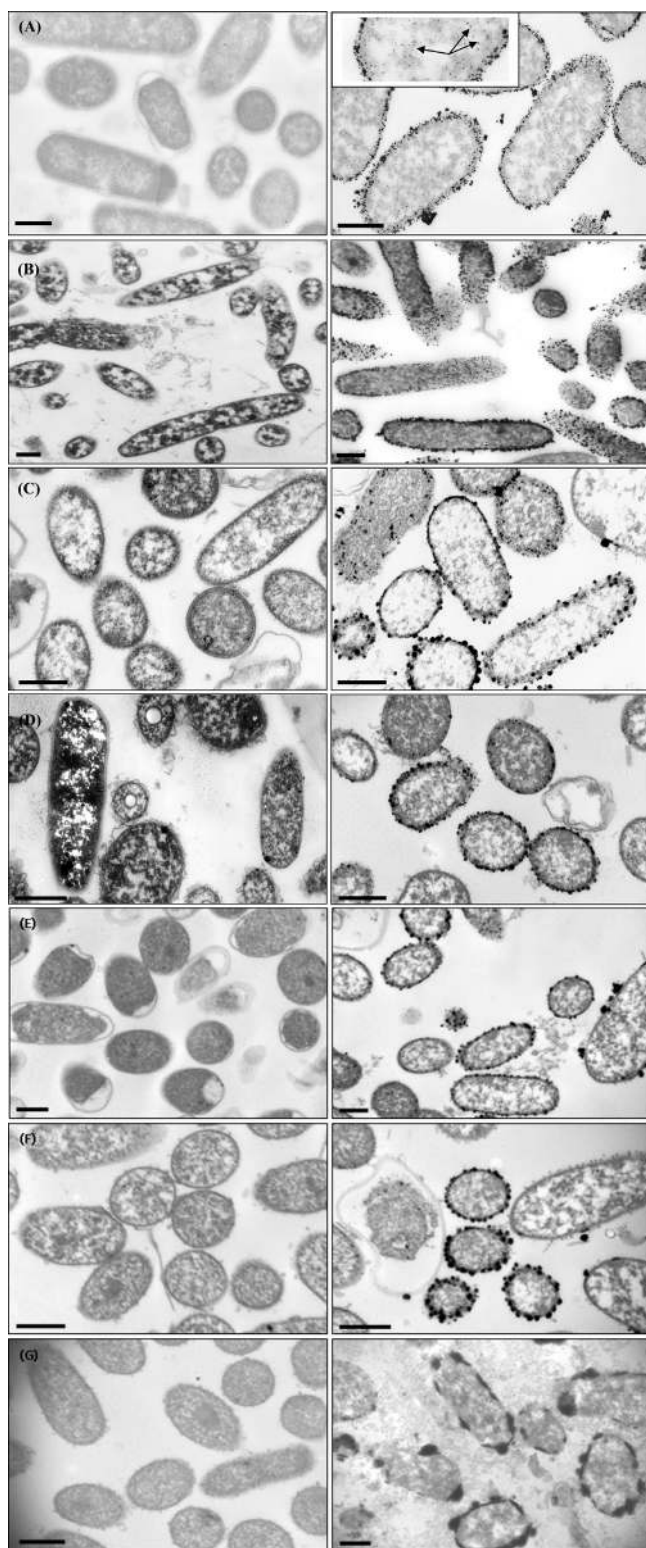


Fig. 2. Electron microscopy of native and palladised strains. TEM micrographs of native (left panel) and palladised (right panel) cells of (A) *E. coli* MC4100 (inset; magnified area of a cell transect), (B) *D. desulfuricans*, (C) *C. metallidurans*, (D) *S. oneidensis*, (E) *Serratia* sp., (F) *M. luteus* and (G) *A. oxydans*. Cells were loaded at 1:3 Pd:dry weight of cells using H_2 as electron donor, harvested by centrifugation and prepared for TEM as described. Scale bars are 500 nm.

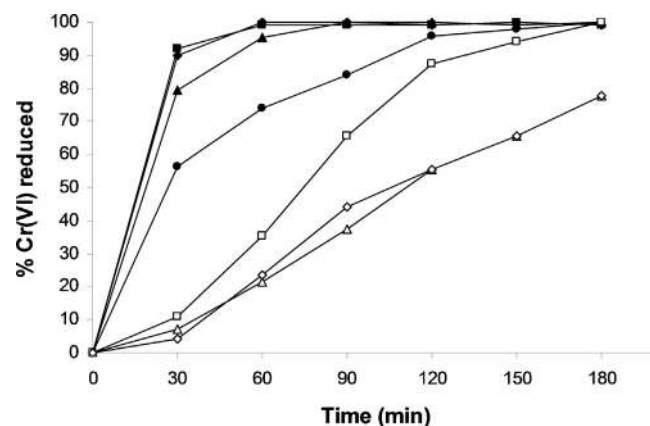


Fig. 3. Catalytic activity of bioPd(0) preparations obtained from various strains in the reduction of Cr(VI). Time course of reduction of 0.5 mM Na_2CrO_4 using 5% bioPd(0) (mass ratio of 1:19 Pd:biomass, 10 mg of catalyst per 12 ml reaction volume). The residual Cr(VI) concentration was estimated at each time point by the diphenyl-carbazide method. Results are expressed as % Cr(VI) reduced over 3 h. (?) *E. coli* MC4100, (i) *D. desulfuricans*, (?) *C. metallidurans*, (?) *S. oneidensis*, (?) *Serratia* sp., (?) *M. luteus* and (?) *A. oxydans*. Data are means \pm SEM from three experiments. Where no error bars are shown, these were within the dimensions of the symbols.

in the gram negative strains which is consistent with the localisation of hydrogenases (and also c_3 -cytochromes), the putative Pd(II)-reducing apparatus [16–18,27]. In addition, close inspection of micrographs of *E. coli* (Fig. 2, inset) suggested the presence of very small (<5 nm) electron opaque cytoplasmic deposits not visible in cells not exposed to Pd(II). Foulkes et al. [39] also observed intracellular Pd deposits using aerobically grown cells, localised mainly in association with the inner membrane, whereas the cells shown in Fig. 2A had small NPs distributed through the cytoplasm.

3.4. Examination by X-ray powder diffraction

Using the two best strains (*D. desulfuricans* and *E. coli*; see later) the X-ray powder patterns of cells loaded at 1:19 with Pd(0) showed the presence of poorly crystalline material consistent with the presence of small nanoparticles. The respective nanoparticle sizes for bioPd_{*E. coli*} and bioPd_{*D. desulfuricans*} were calculated from the X-ray diffraction data using the Scherrer equation as 6.1 ± 0.8 nm and 3.34 ± 0.09 respectively, which is comparable to NPs of bioPd_{*D. desulfuricans*} found previously as 4.7 nm using magnetic measurements [9] and 5 nm by direct measurement of isolated NPs using high resolution TEM [10]. The geometry (1 1 1) and (2 0 0) was consistent with icosahedral NPs but distorted with surface imperfections [10] postulated to underpin enhanced catalytic activity as compared to a colloidal homogenous catalyst [10].

3.5. Catalytic testing of bioPd made from various strains

3.5.1. Cr(VI) reduction

Fig. 3 compares the catalytic activity of the bioPd preparations in the reduction of Cr(VI) (CrO_4^{2-} ; aq). All bioPd catalysts were active but with variable initial Cr(VI) reduction rates. With over 90% Cr(VI) reduced after 30 min, bioPd_{*D. desulfuricans*} and bioPd_{*E. coli*} showed the most rapid initial rate and similar overall catalytic activities; bioPd_{*Serratia*} sp. was slightly slower. Slow rates were seen using bioPd_{*C. metallidurans*}, bioPd_{*A. oxydans*} and bioPd_{*S. oneidensis*} and, with the latter two, Cr(VI) reduction did not go to completion over the reaction period (180 min). Although bioPd_{*M. luteus*} gave a faster initial rate than these three bioPds the overall reaction proceeded slowly, with complete Cr(VI) reduction observed after 180 min (similarly to bioPd_{*C. metallidurans*}) whereas complete Cr(VI) reduction was observed within 60 min in the best cases. There was no correlation

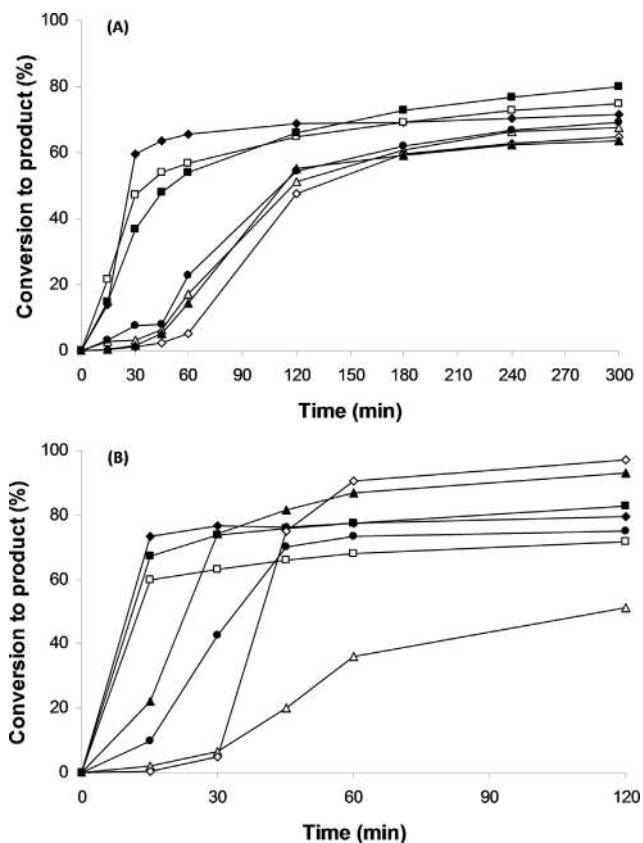


Fig. 4. Catalytic activity of bioPd(0) preparations obtained from various strains in the Heck reaction. Conversion to product (%) in the coupling of (A) phenyl iodide and styrene and (B) phenyl iodide and ethylacrylate over bioPd(0) catalysts (10.6 mg of 5% bioPd(0)), 20 ml reaction volume) made from (?) *E. coli* MC4100, (†) *D. desulfuricans*, (?) *C. metallidurans*, (?) *S. oneidensis*, (?) *Serratia* sp., (?) *M. luteus* and (?) *A. oxydans*.

between Pd(II) uptake capacity or Pd(II) bioreduction rates and Cr(VI) reduction rates (r^2 of 0.036 and 0.147 respectively; Table 3).

The intuitive relationship between catalytic activity and the NP size on the cells (i.e. available surface area) was shown previously comparing bioPd_{*D. desulfuricans*} and bioPd_{*E. coli*} at loadings of 1:3 by mass [38]; a relationship between the NP size on Gram positive and Gram negative cells (Fig. 2) is consistent with the relative catalytic activities shown in Fig. 3.

3.5.2. Comparison of the bioPd of the strains in the Heck coupling

Fig. 4 shows % conversion to product in the Heck coupling of phenyl iodide (PhI) and styrene (Fig. 4A) and PhI and ethylacrylate (Fig. 4B) in dimethylformamide. All bioPd catalysts were active in both reactions but with variable reaction rates. With the exception of bioPd_{*A. oxydans*} which showed low activity in the coupling of PhI and ethylacrylate (51% conversion), the overall conversion for each reaction was comparable ranging between 65 and 80% and 73 and 82% for coupling of PhI and styrene and PhI and ethylacrylate respectively.

The rate of Heck coupling over bioPd catalysts was biphasic in some cases and the reaction profile was not the same for all. In both reactions, bioPd_{*E. coli*}, bioPd_{*C. metallidurans*} and bioPd_{*D. desulfuricans*} showed a rapid primary phase and a slower secondary phase. The order of catalyst activity was, overall, similar in both reactions, with bioPd_{*E. coli*} effective in both cases, with the fastest initial reaction rate and 75% conversions. BioPd_{*D. desulfuricans*} and bioPd_{*C. metallidurans*}, although slightly less active, had comparable overall catalytic activity while bioPd_{*A. oxydans*} was consistently poor. BioPd_{*M. luteus*}, bioPd_{*Serratia*} and bioPd_{*S. oneidensis*} were inconsistent, giving better

coupling of PhI and ethylacrylate than of PhI and styrene. The latter showed a unique reaction profile in the Heck coupling of PhI and ethylacrylate: a 30 min lag phase was followed by reaction at a high rate to reach 90% conversion. Conversion to product over bioPd_{*Serratia*} and bioPd_{*S. oneidensis*} catalysts (both from gram negative cells) showed opposite trends over the two reactions: while the latter was the highest in PhI and ethylacrylate (>90% conversion), it was amongst the lowest of the bioPd catalysts for the coupling of PhI and styrene (60% conversion). No correlation between Pd(II) uptake/bioreduction and activity of bioPd catalysts in Heck coupling reactions was established although there was a correlation between the activities in the two Heck reactions (Table 3).

3.5.3. Selection of bioPd for further evaluation

The 7 strains were ranked without weighting using 6 criteria: i.e. reaction rates and conversions in the 3 reactions, shown in Figs. 3 and 4 (Table 4). The Gram positive strains were consistently ranked low and were not considered further. BioPd_{*D. desulfuricans*} and bioPd_{*E. coli*} were in the top 4 positions considering both criteria.

The final selection was made on the basis of several additional criteria. *D. desulfuricans* produces H₂S, a powerful catalyst poison, during its pre-growth, requiring extensive washing of the cells prior to making bioPd; insufficiently washed cells showed traces of PdS by XRD (I. Mikheenko, unpublished). The final cell yield of anaerobic bacteria is low; hence large scale growth may be problematic. In contrast *E. coli* does not produce catalyst poison and can be pre-grown to high cell density aerobically and then placed anaerobically for induction of its anaerobic enzymes; in fact, even well mixed high density cultures have sufficiently low oxygen transfer to enable effectively a constant switching between aerobic and anaerobic metabolism in ostensibly aerobic culture [40]. Furthermore, 'waste' *E. coli* cells from a primary process such as hydrogen production [34] or therapeutic protein production (see [41]) can be used subsequently for manufacture of catalytically active bioPd following a primary fermentation (e.g. from H₂ production [14,34]). Finally, the use of *E. coli* presents wide opportunities for rational design of the biocatalyst material using the well developed molecular engineering tools for this organism, targeting the cellular processes by which bioPd is produced (e.g. [14]). Moreover, preliminary studies showed biofilm formation of *E. coli* onto solid support (C. Mennan, I. Mikheenko and L.E. Macaskie, unpublished) via the same method as that described for *Serratia* sp. facilitating potential development of an immobilised catalyst [12,38]. In contrast, a cohesive biofilm of *Desulfovibrio* was not produced [31].

3.6. Further evaluation of BioPd_{*E. coli*}

In order to more fully evaluate the bioPd_{*E. coli*}, its use was assessed in a standard Heck reaction between phenyl iodide and ethyl acrylate under two sets of conditions (see Table 5a) including known literature conditions for carrying on a Heck reaction using a supported Pd(0) catalyst in the absence of a ligand [42]. Both sets of conditions gave comparably good yields in this reaction (75% and 78% respectively). BioPd was shown previously as comparable to both a homogeneous colloidal Pd catalyst and a commercial 5% Pd on carbon catalyst [10].

The catalysts were further assessed on a selection of alternative Heck substrates to determine their applicability. Activity was demonstrated on the substrate 4-bromoacetophenone, however, 3-chlorotoluene failed to react under these conditions.

The scope of the bioPd_{*E. coli*} was then extended to other palladium-catalysed reactions. To this purpose a number of substrates were screened in another vital C–C bond reaction in chemical synthesis, the Suzuki reaction, in this case with phenyl boronic acid as the coupling partner (Table 5b). In contrast to the Heck reaction using this catalyst, it was found necessary to

Table 4
Selection of microbial Pd(0) support for further evaluation Rank reaction.

Rank order	Reaction					
	Cr(VI) reduction (Fig. 3)		Heck coupling (Fig. 4a)		Heck coupling (Fig. 4b)	
	Rate	Conversion	Rate	Conversion	Rate	Conversion
1	Dd/Ec	Dd/Ec/Ser/MI/Cm	Ec	Dd	Ec	So
2			Cm	Cm	Dd	Ser
3	Ser		Dd	Ec	Cm	Dd
4	MI		MI	MI	Ser	Ec
5	Cm		Ao/Ser	Ao	MI	MI
6	So	So/Ao	So	So	So	Cm
7	Ao		So	Ser	Ao	Ao
Occurrences in the top 4 positions		Dd (6) Ec (6) Cm (4) Ser (4) MI (3) So (1) Ao (0)	Occurrences in the top 2 positions:		Dd (4) Ec (4) Cm (2) Ser (2) MI (1) So (1) Ao (0)	

Dd, *D. desulfuricans*; Ec, *E. coli*; Cm, *C. metallidurans*; Ser, *Serratia* sp.; So, *S. oneidensis*; MI, *M. luteus*; Ao, *A. oxydans*. Bold: Gram positive strains.

employ a phosphine ligand for this reaction, namely the Buchwald monophosphine ligand John-Phos [43,44]. In the absence of this ligand no reaction was observed (Table 5b, Reaction 6). It is possible that the need for a ligand is due to the palladium leaching from the surface in a soluble active species in order to carry out the catalysis, before being re-deposited on the surface of the *E. coli*, as has been postulated for some Pd/C catalysed reactions [45,46].

It was found that the highly activated substrate, 1-bromo-4-(trifluoromethyl)benzene gave excellent conversion under these conditions (Table 5b, Reaction 7), while the catalyst also displayed good activity towards electron-rich aryl bromides such as 4-bromoanisole (Table 5b, Reaction 5), although surprisingly no reaction was seen with 2-bromopyridine under these conditions. Interestingly, the activated substrate 4-bromoacetophone gave only moderate conversion in this reaction (Table 5b, Reaction 8), however, this phenomenon has been described previously in several studies [47–49]. As in the Heck reaction, the aryl chlorides were found to be less reactive under our conditions, with 4-chloroanisole giving only 17% yield, and 3-chlorotoluene resulting in no product formation. However, it must be noted that the palladium loading for the Suzuki reactions carried out was only 0.1 mol%, compared with 0.3 mol% for the Heck reactions. Hence, overall, the BioPd catalyst is shown to be a viable recoverable [10] heterogeneous catalyst for two key C–C bond forming reactions, at very low loadings of palladium.

4. Discussion

In this study an *E. coli*, *D. desulfuricans*, a *Micrococcus* sp. and *S. oneidensis* showing hydrogenase-mediated metal reduction [4,6,50–52] were tested for their ability to reduce Pd(II). *M. luteus*, *A. oxydans* and *C. metallidurans* often show high tolerance to a large number of metallic ions [53–60] and actinides [61] by mechanisms which include efflux systems, biosequestration and/or bioprecipitation [54,56,57] prompting their inclusion in the study. The high ability of *C. metallidurans* to accumulate a broad range of heavy metals is well known [57] (and references therein) and has been linked to the presence of two megaplasmids (*pMOL28* and *pMOL30*). In addition, this bacterium possesses multiple efflux pumps on the cell surface; von Rozycki et al. [62] showed that, in *C. metallidurans*, 13% of the total protein content comprises putative transporters of metal ions and organic molecules, which is more than in any other fully sequenced micro-organism. The strain of *Serratia* NCIMB 40529 (a close relative of *E. coli*) was included in the study because it

has been shown to make bioPd active in Cr(VI) decontamination in a continuous flow-through column when immobilised as a biofilm [12,24].

The archaea were omitted from this investigation since methanogens require specialist culture conditions and were found to make bioPd of unexceptional catalytic quality [63]. Hyperthermophiles, although often having exceptionally high hydrogenase activities (e.g. *Pyrococcus furiosus*), could not be grown in sufficient quantity for testing (K. Deplanche, unpublished) and the use of halophiles would tend to shift the solution chemistry of Pd completely into formation of complexes like PdCl_4^{2-} , negating the constant chemical conditions applied in the biomanufacturing system.

We show that Pd(II) accumulation by *M. luteus* and *A. oxydans* during the pre-incubation period ('biosorption') was inferior to passive Pd(II) uptake by all gram negative strains. This is surprising as many studies have highlighted the high capacity of gram positive bacteria towards metal biosorption (e.g. [64,65]). *M. luteus* is a known effective biosorbent in the recovery of Co^{2+} , Cu^{2+} , Pb^{2+} , Ni^{2+} and Zn^{2+} from aqueous solutions [66] and acid-mine drainage (AMD) water [59], while the ability of *A. oxydans* to accumulate both CrO_4^{2-} and Ni^{2+} is well documented [60] and suggests both negatively and positively charged cell surface groups although in the current study at pH 2.3 cell surface protonation is likely. Pd solution chemistry dictates that the free Pd^{2+} ion is rarely found in solution. Pd(II) (Na_2PdCl_4) mainly exists in the form of the $[\text{PdCl}_4^{2-}]$ complex [3] which coordinates readily to protonated biomass carboxyl and amine groups at pH 2.3 [28].

During all steps of bioPd manufacture, the fate of soluble Pd(II) ions was monitored (Table 2 and Fig. 1). Yong et al. [20] showed that a 'biosorption' step was required to obtain bioPd preparations with maximal catalytic activity and, while possibly circumstantial, the biosorption of Pd(II) by *D. desulfuricans* was observed to be 1.7-fold higher than that of *D. vulgaris* and *D. fructosovorans* [28] and the former strain produced bioPd that was 1.8-fold more active in dehalogenation of 2-chlorophenol [67] suggesting a dictation of the initial biosorption into the 'quality' of the bioPd produced. This was confirmed in this study; the uptake of Pd(II) during the initial 'passive' uptake correlated with the catalytic activity of the resulting bioPd, attributed via electron microscopy to the initial formation of nuclei at many coordination sites, and hence smaller nanoparticles per given mass of Pd(0). The latter was also suggested previously in the reduction of Cr(VI) and dehalogenation of chlorinated aromatic compounds by bioPd_{*D. desulfuricans*} and bioPd_{*E. coli*}

Table 5
Industrial testing of catalytic activity of 2.5% bioPd_{E.coli} in Heck and Suzuki couplings.

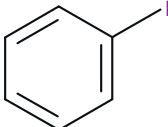
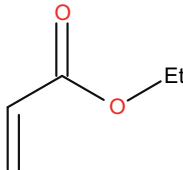
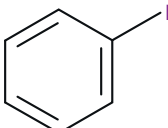
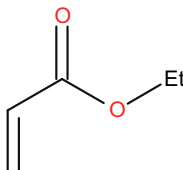
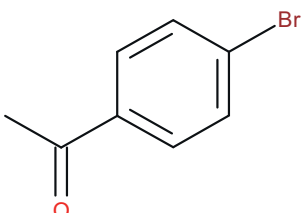
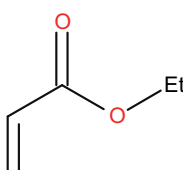
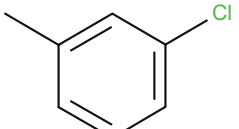
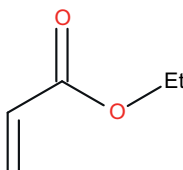
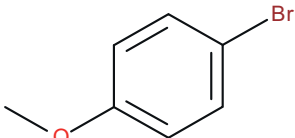
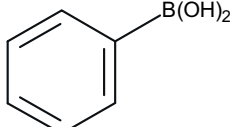
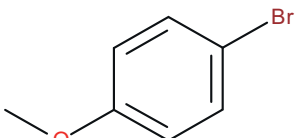
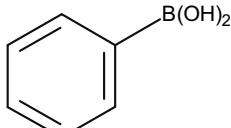
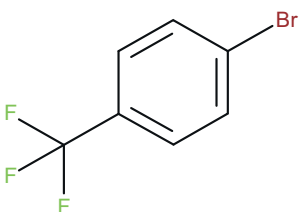
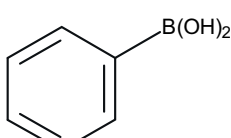
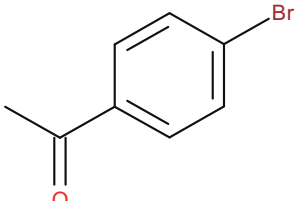
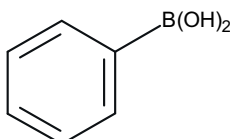
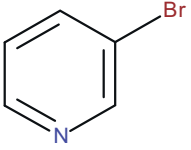
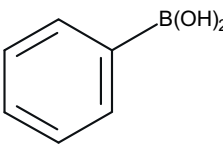
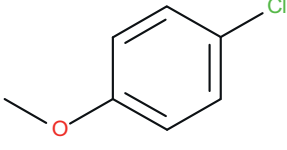
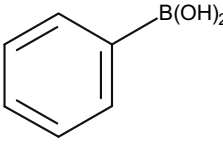
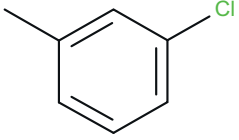
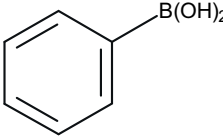
No.	Aryl Halide	Coupling partner	Pd loading (mol%)	Rxn time	Yield (%) ^a
(a) Heck reaction					
1			0.3	2 h	78
2			0.5	2 h	75 ^{b,c}
3			0.3	5 h	54
4			0.3	5 h	0
(b) Suzuki reaction					
5			0.1	18 h	62
6			0.1	18 h	0 ^d
7			0.1	18 h	90
8			0.1	18 h	23 ^e

Table 5 (Continued)

No.	Aryl Halide	Coupling partner	Pd loading (mol%)	Rxn time	Yield (%) ^a
9			0.1	18 h	0
10			0.1	24 h	17
11			0.1	18 h	0

Conditions for Heck reaction: aryl halide (3.0 mmol), ethyl acrylate (3.0 mmol), 2.5 wt% bioPd(0)/*E. coli* (0.3 mol%), Na₂CO₃ (3.0 mmol), NEt₃ (3.0 mmol), NMP (15 mL), 110–130 °C. Conditions for Suzuki reaction: aryl halide (3.0 mmol), phenyl boronic acid (3.6 mmol), 2.5 wt% bioPd(0)/*E. coli* (0.1 mol%), John-Phos (1 mol%), Na₂CO₃ (6.0 mmol), EtOH (9 mL), H₂O (3 mL), 80 °C.

^a Isolated.

^b Lab scale Heck conditions (Section 2.7); aryl halide (1.0 mmol), ethyl acrylate (1.5 mmol), 5 wt% bioPd(0)/*E. coli* (0.5 mol%), NEt₃ (1.5 mmol), DMF (15 mL), 120 °C.

^c conversion calculated from HPLC.

^d Run in the absence of John-Phos ligand.

^e Conversion calculated from ¹H NMR.

[38]. This study expands this concept from the environmental focus of previous work to the study of two couplings of key relevance to the green synthesis of fine chemicals.

The high catalytic activity of biogenic Pd nanoparticles (NPs) is now very well reported by many authors since the initial reports of Mabbett et al. [68] (reductive dehalogenation) and Mabbett and Macaskie [69] (Cr(VI) reduction) (see reviews [7,24,25]). The structure of individual Pd-bionanoparticles, (of size 5 nm; confirmed by XRD in this study as well as magnetically [70]) revealed irregular crystal faces and other surface features which may be responsible for enhanced activity as compared to a commercial catalyst in the Heck reaction [10]. Notably bioPt NPs that were cleaned of their residual biological material showed increased resolution in cyclic voltammograms, i.e. progressive unmasking of the metal surface was accompanied by an altered reaction specificity as the biochemical component was removed [71]. The degree of NP agglomeration in nascent cleaned NPs was not determined but it was suggested experimentally (by monitoring bio-NP size during sequential catalyst recovery and re-use [10] that a key role of the biomatrix is to confer stability against agglomeration. In accordance with the direct tests using 'cleaned' material (above) the supporting/residual biomatrix is known to play a role in reaction specificity via steering the Pd-NP location into alternative sites (i.e. aqueous periplasmic polymeric gel or phospholipid membranes) by use of specific mutations [17,18]. However, to date no studies have attempted to determine the influence of the biological support on the activity of the resulting catalyst in a structured way in reactions involving inorganic and organic substrates and/or taking place in aqueous solutions or in solvent.

Initially Cr(VI) reduction was examined in aqueous solution with a view to decontamination of aqueous toxic wastes for environmental protection (see [24]); the ability of bioPd to decontaminate real groundwater with respect to chlorinated aromatic compounds was shown elsewhere [72].

This study shows that the best catalyst in aqueous solution was that supported on the gram negative surfaces although that

of bioPd_{C. metallireducens} was inferior to the other gram negative strains. Here, most of the Pd was deposited before addition of H₂. It has been affirmed [21], following pioneering work using extracted native and functionalised chitosan biopolymers [73,74], that passive uptake onto biomass functional groups followed by chemical reduction of sorbed Pd(II) to Pd(0) produces bioPd without a mandatory need for an enzymatic contribution. Indeed, the *C. metallidurans* preparation turned black instantly upon addition of H₂ suggesting a possibly predominant chemical reaction; however this, and the possibility of endogenous partial reduction, e.g. to a form of Pd(I) was not confirmed. It could be suggested that some catalytic potential of the bioPd may be lost by using a 'chemical' rather than a hybrid chemical/biochemical approach; other tests confirmed that a hydrogenase-deficient mutant of *E. coli* (i.e. in this case passive uptake of Pd(II) followed by chemical reduction) fabricated bioPd which reduced Cr(VI) at a rate >15-fold less than the hydrogenase-containing parent strain [18].

C. metallidurans interacts with soluble Au(III) via formation of biogenic Au(I)-sulfur complexes [75] but the mechanism of interaction of cells with Pd(II) is unknown. Since sulfur is a potent Pd-catalyst poison the same mechanism for sequestration of Pd might be expected to produce a Pd catalyst of low potential (as seen here with CrO₄²⁻). However bioPd_{C. metallidurans} was found to be active in the Heck coupling (Fig. 4) which introduces a concept of reaction selectivity with respect to substrate and solvent by partial catalyst sulfur-poisoning, an approach appreciated in industrial synthesis but not extensively implemented at commercial scale due to difficulties of reproducibility (B. Murrer, Johnson Matthey, personal communication). As examples, natural S-poisoning of a chemical Pd catalyst treating chlorinated hydrocarbons in groundwater gave a 30% reduction in conversion but increased the reaction specificity [76] while a Pd catalyst coated with n-alkanethiol showed a reduced rate but the selectivity in the hydrogenation of 1-epoxy-3-butane to 1-epoxybutane was increased from 11% to 94% [77]. Clearly a more detailed study of bioPd_{C. metallidurans} is warranted as a first putative example of the potential ability of

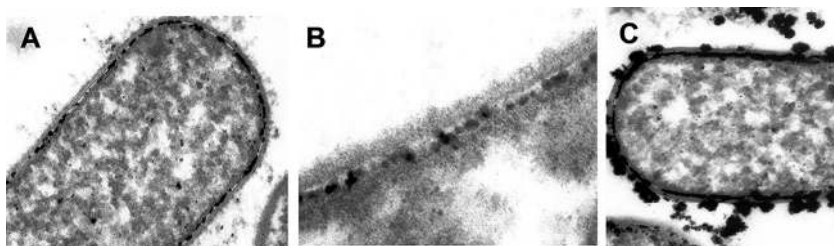


Fig. 5. Electron microscopy of palladised bacteria. BioPd made on *Bacillus sphaericus* by the standardised method described in this study. Adapted from Skibar et al. [31].

microorganisms to 'steer' catalytic activity via bioligand intervention.

With the dual goals of potential reduction in consumption of primary metal reserves and green chemistry with respect to increased reaction rate/specificity by a recoverable, re-usable catalyst [10], the activity of Pd NPs supported on cells of *D. desulfuricans*, *B. sphaericus* [9], and various other organisms [7,24,25] has been described previously but not comparatively and the literature now burgeons with examples of Pd-bionanocatalysts. However differences in catalyst preparation, testing conditions and solvents complicate a comparison of catalytic data. Therefore this study standardised the conditions of bioPd manufacture in order to carry out a comparison of bioPd made from several strains in order to indicate which generic types of bacteria (i.e. gram positive and gram negative) make active bioinorganic Pd catalysts. *Bacillus* (gram positive) was not included since a bioPd_{*B. sphaericus*} has been the subject of extensive previous work, including a comparative hydrogenation, where it made bioPd of comparable activity to bioPd on gram negative cells [9]. Being obligately aerobic, *Bacillus sphaericus* used in this study would have had no hydrogenase activity expressed and in this case a dual mechanism could involve Pd(0) deposition onto the protein [29] and also within the peptidoglycan layer via chemical reduction [30,78].

A previous study not formally published [31] showed that, under the conditions used here, with *B. sphaericus* (now *Lysinibacillus sphaericus*) there was no Pd(0) visible in the outermost S-layer but Pd-nanoparticles were clearly apparent beneath this, within the peptidoglycan layer (Fig. 5A and B; reproduced from [31] which appeared similar to the appearance of the gram negative cells of bioPd_{*D. desulfuricans*} and bioPd_{*E. coli*} (Fig. 2A and B) and gave similar catalytic activity to bioPd_{*D. desulfuricans*} in the hydrogenation of itaconic acid [9]. However at a higher pH (3.4) bioPd was made as larger particles on the cell surface [31], similar to those seen in the gram positive strains used in this study (Fig. 2F and G) and, accordingly, the bioPd_{*B. sphaericus*} shown in Fig. 5C had 20% lower activity in reduction of Cr(VI) than that shown in Fig. 5A and B [31] (c.f. Fig. 3). Extensive speculation is unwarranted except to note that this strain of *B. sphaericus* was originally isolated from a site contaminated by acidic mine waste pH 4.6 [79] and its S-layer (which was distinct from the S-layer protein of related bacteria) bound cationic metals extensively via phosphate groups due to extensive phosphorylation of the S-layer protein (see [80,81]).

Bacterial S-layers self-assemble; the protein monomers dissociate at high or low pH values. The isoelectric point (*pI*) of S-layer proteins from neutrophilic strains is weakly acidic (due to a predominance of acidic amino acids); strains occupying acidic niches have typically higher *pI* values of their S-layer proteins [82] and an upshift of the *pI* has been suggested as an adaptation to an acid 'lifestyle' [83]. Hence, the S-layer of acid tolerant *B. sphaericus* at acidic pH may be relatively closer to its *pI* as compared to neutrophilic strains. Hence we may assume that, even if S-layers were originally present in *M. luteus* and *A. oxydans*, these would

disassemble at the low pH used here. Additionally, the contribution of the *B. sphaericus* S-layer phosphoryl groups also needs to be taken into account; the third ionisable group of phosphate has a *pK_a* of 2.15 (c.f. that of aspartate and glutamate, the main acidic amino acids of S-layers are 3.65 and 4.25 respectively) and hence the S-layer of this strain is even more likely to maintain integrity under the challenge conditions due to the additional stability and localised buffering effect of its phosphate substituents. This argument, together with the evidence shown in Fig. 5B suggests low, if any, involvement of S-layers in bioPd formation by *M. luteus* and *A. oxydans* under these conditions. Inspection of Fig. 5B shows clearly that the Pd(0) layer was located beneath the S-layer in *B. sphaericus*, i.e. Pd(II) had passed through it. In this organism the S-layer protein is anchored to a secondary cell wall polymer comprising N-acetyl glucosamine and N-acetyl mannosamine, every second unit of which carries a pyruvate ketal which confers a net negative charge (see [84]) and hence sites for Pd(II) coordination but the peptidoglycan layer is relatively thin in *Bacillus* [85]. Biomanufacture and evaluation of catalytic activity of bioPd made by various *Bacillus* spp., including those that do not produce S-layers (e.g. *B. subtilis*, *B. megaterium*, *B. licheniformis* [85]) is the subject of another investigation and will be reported subsequently.

Beveridge [85] compared the cell surface of *B. brevis* and *Arthrobacter globiformis*, noting the absence of S-layer proteins from the latter, and also that *A. globiformis* and *M. luteus* have thick, robust cell wall peptidoglycan layers whereas *Bacillus* spp. have thinner peptidoglycan layers. The current study (from the above) assumes the mechanism of Pd-deposition in *A. oxydans* and *M. luteus* to be nonspecific or peptidoglycan-mediated. Beveridge [85], while investigating the root of gram-variability, noted that as gram positive cultures age during exponential growth individual cells fragment their peptidoglycan layer and die, which is a possible reason for the high number of apparently Pd(0)-free cells of *M. luteus* apparent in Fig. 2F.

It was also noted [85] that exudates produced by *Arthrobacter* caused the cells to grow as flocs, in accordance with the high amount of extracellular polymeric material visible in Fig. 2G. We observed that, during the passive Pd(II) uptake, both of the gram positive strains showed a tendency to flocculate and form a heavy precipitate with Pd(II) (even with mixing) while the gram negative strains remained evenly dispersed. It is possible that, upon addition of Pd(II) solution, the former were more importantly affected by the sudden change of pH. In wastewater treatment (activated sludge process), a decrease of the pH during anoxic settling promotes flocculation, the precise mechanism of which is still conjectural [86] and references therein) but is probably associated with cross-linking of microbial extracellular polymers. In this context, the cells of *A. oxydans* following Pd(II) exposure appeared to be held within a matrix of extracellular material which became electron opaque in the presence of Pd(II) (Fig. 2G). In comparison, the gram negative *Serratia* sp. NCIMB 40259 is well documented to produce copious extracellular polymer (the

root of its ability to produce a cohesive biofilm) [87], however agglomeration of cells of this strain has not been observed and no large Pd-NPs were seen on addition of H₂ although other studies using *Serratia* sp. extracted polymer showed by electron microscopy and NMR that addition of Cd(II) promoted crosslinking and EPM agglomeration [88]. It is suggested that the agglomeration reported here for *A. oxydans* at acidic pH may have been due to a similar crosslinking via Pd(II) but this was not investigated further.

In contrast to the gram positive strains, the gram negative strains (apart from *C. metallidurans*: see above) gave consistently active bioPd. The mechanism of its synthesis, anaerobically, is now shown to involve hydrogenases (see earlier). Hydrogenases catalyse the reversible reduction of protons into molecular hydrogen and are pivotal in the energy generation processes of many microorganisms. They are classified according to the metal content of their active site and their role in uptake or formation of hydrogen. [Fe-Fe] hydrogenases are usually H₂-forming while [Ni-Fe] hydrogenases can function in either H₂ uptake and splitting or in H₂ release; the turnover of the former is 100-fold higher than the latter [89]. These enzymes are usually expressed only under anaerobic conditions and are typically highly sensitive to oxygen although the membrane bound [NiFe] hydrogenase of *Cupriavidis* sp. is tolerant to ambient O₂ levels [90], while a pathogenic *Salmonella* species (but not *E. coli*) has a newly described hydrogenase which functions in aerobic hydrogen metabolism [91].

The ability of hydrogenases to function as broad-specificity metal reductases is now well established [50,92–95]. The precise mechanism of Pd(II) bioreduction is still not fully elucidated but several studies have highlighted the key role of bacterial hydrogenases in the initial formation of Pd(0) seeds (nucleation step) [17,18,27] following which Pd(II) reduction is thought to be autocatalytic [19] with the events and cellular localisation of initial Pd(0) deposition and crystal growth influencing the patterning, structure and activity of the finished catalyst. Recent studies have affirmed the concept of biosorption, nucleation and autocatalytic NP manufacture [21].

However, use of an enzymatic mechanism offers the potential for 'fine control'. Two separate studies showed that deletion of specific hydrogenases can affect the catalytic activity of the resulting bioPd. Although deletion of the periplasmic [Fe]-only hydrogenase from cells of *D. fructosovorans* did not affect the rate of Pd(II) reduction, the resulting bioinorganic Pd catalyst was more active in the reduction of Cr(VI) [26]. In similar studies using deletion mutants of *E. coli*, the presence of the membrane-bound, periplasmic facing H₂-consuming [Ni-Fe] hydrogenase was required to obtain a catalyst with comparable activity to the wild type [18].

The free Pd²⁺ ion is rarely found in solution (see above). Pd(II) coordinated readily to partially protonated carboxyl and amine groups of *D. fructosovorans* at pH 2.3 [28] and it is likely that the difference in biosorption behaviour between strains (Table 2) was attributable to specific biomass ligands available for initial Pd(II) binding under these specific conditions. Thus our data (Fig. 1) suggest that, under the standardised conditions, passive uptake of soluble Pd(II) species is more readily achieved by gram negative strains.

However a major gap in our understanding lies in knowing the mechanism of transport of incoming Pd(II) from the initial 'biosorption' sites to its final close proximity to the hydrogenase(s) (see earlier). In anaerobically grown *E. coli* the bulk of the Pd(II) reducing activity was attributable to the hydrogenases located in the inner membrane [18], requiring Pd(II) to traverse a distance across the periplasmic space of 30 nm followed by its transport to the membrane-bound hydrogenase itself. Further 'Pd-trafficking' across the cytoplasmic membrane via an unidentified mechanism is implied also by the observation of small Pd-NPs in the cell cytoplasm (Fig. 2A). Beyond the remit of this work, but possibly key

to our understanding of the interaction between Pd(II) and hydrogenase enzyme, and the resulting high-activity Pd-NPs, is that Pd and Ni, in the same group of the periodic table, share some chemical similarities [96]. Hydrogenase is one of the few enzymes that requires Ni, a toxic metal. Hence, an efficient microbial Ni trafficking mechanism is anticipated. In their elegant review of how cells ensure that metalloproteins receive the correct metal, Waldron and Robinson [97] suggested that Ni supply via the NikA-E importer is 'hardwired' to hydrogenase. Further, the protein SlyD, with a carboxyl rich region, is thought to act as a Ni reservoir, receiving Ni delivered directly from NikA-E [97]. If the Ni(II) trafficking system is indeed implicated in Pd(II) targeting to hydrogenase this would explain how Pd(II) can be 'delivered' to the enzyme. Note that this study used resting cells at pH 2.3 at which pH the activity of pre-formed hydrogenase activity is maintained for only a limited period [17]; it could be assumed that the cells may be 'attempting' to replenish active hydrogenase enzyme while suffering acid-stress and while being physiologically starved of Ni(II) in the presence of excess Pd(II).

The bioPd preparations made from *E. coli* and *D. desulfuricans*, which both have high hydrogenase activities and which both showed a similar homogeneous coverage of Pd(0) particles (Fig. 2B, 2D), showed the best catalytic activity (initial reaction rate) in all three of the test reactions (Figs. 3 and 4). Although Cr(VI) reduction and Heck coupling over Pd(0) catalysts would involve inherently different reaction mechanisms (one is addition of electrons to CrO₄²⁻, the other is hydrogenolysis of the carbon-halide bond), the overall activity of their bioPd catalysts appeared to be fairly consistent across all test reactions. In contrast bioPd_{*C. metallidurans*} (see earlier) and bioPd_{*Serratia*} showed a significant difference in performance between the two type of reaction, performing significantly better in the Heck reaction in solvent than in Cr(VI) reduction (aq.). This might be attributable to differences in their bioPd 'fitness' for different reaction mechanisms. Cr(VI) reduction relies on the ability of Pd(0) to (i) trap reactive H• via homolytic cleavage of the electron donor molecule (exogenously supplied formate) and (ii) to act as a surface for the transfer of electron from H• to CrO₄²⁻ (see [98] whereas the Heck coupling is a more complicated reaction involving changes in the state of the Pd itself (see below).

In this study *E. coli* was pre-grown anaerobically (expressing hydrogenases) whereas its close relative *Serratia* was grown aerobically. Notably, in the present study the *Serratia* sp. produced catalytically active bioPd even though aerobically grown cells do not normally express hydrogenase. In accordance with the conclusion of Deplanche et al. [18] using a hydrogenase deficient *E. coli*, it is clear that a second mechanism of Pd(0) deposition is involved.

The mechanistic process of the Heck reaction is fairly well understood and involves a more complex cascade of reactions as detailed elsewhere [99]. In his initial studies, Heck noted that Pd/C catalysts (i.e. Pd(0) supported on activated carbon) had low activity compared to soluble Pd complexes (Pd chloride/acetate, triphenyl-phosphine) and, to date, the commercial Heck reaction still belongs to the domain of homogeneous catalysis [100]. The high biosorption capacity of *C. metallidurans* biomass might have led to the presence of unreduced Pd(II) (or single Pd atoms) near the cell surface which could have proved beneficial in the Heck reaction but without benefit in Cr(VI) reduction. The differences in catalytic activity exhibited by bioPd_{*S. oneidensis*} and bioPd_{*Serratia*} across both Heck couplings suggests the existence of several models of biological NPs passivation as Pd deposits from each strain appeared similar in size and dispersion (Fig. 2D and E). It is worth noting that, while using bioPd to reduce Au(III) to Au(0) [101], bioPd reoxidises and re-reduces (under H₂) at the surface of the newly formed bioAu NP to form a core shell structure with some Pd detected external to the NP itself. The net result is a migration of Pd(0) from the cell surface to the shell of the NP but whether this

was by 'recapture' of soluble Pd(II) ions or by 'creeping around' the NP was not established.

This study not only clearly demonstrates the influence of the biological support matrix in the synthesis and passivation of biogenic Pd NPs but it raises clear questions about the mechanisms of Pd(0) biomanufacture in the different strains. Although good correlation was observed between the initial rate of Pd(II) bioreduction and the amount of Pd(II) removed during the uptake phase, attempts to correlate the results of catalytic testing with various parameters of bioPd manufacture were unsuccessful (Table 3).

However, since a similar reaction mechanism is involved, bioPd active as a Heck catalyst should also be useful in the Suzuki reaction. Indeed the BioPd catalyst was shown to be a viable heterogeneous catalyst for both of the key C–C bond forming reactions assessed (Heck and Suzuki reaction), at very low loadings of palladium (0.3 and 0.1 mol% respectively).

The development of an active heterogeneous Pd catalyst for the Heck and Suzuki coupling reactions is of significant interest [102]. Palladised cells of *E. coli*, *D. desulfuricans* and *C. metallidurans* all showed high activity in the Heck coupling of phenyliodide and ethylacrylate. This represents an industrially relevant advance since the Heck and Suzuki couplings are key bond-forming reactions used in the chemical industry. As bioPd catalysts can be manufactured from the biorecycling of PGM-containing wastes [7,8,103] some catalysts of which have greater activity than the corresponding catalyst made from pure metal (see [7]) the manufacture of low-cost Pd catalysts is a distinct move towards green chemistry for industrial synthesis, while the potential imposition of reaction selectivity via understanding and selection of a particular cell type for a particular reaction is a major advance beyond the simple biogenesis concept and moves towards molecular engineered catalyst design.

Acknowledgements

This work was supported by the BBSRC (Grant No. BB/C516128/1), EPSRC (grants no EP/I007806/1 and EP/D05768X/1) and by a British Council Entente Cordiale fellowship to K.D. L.E.M. was supported by a BBSRC/Royal Society Industrial Fellowship. We thank Mr. Graham Burns (School of Chemistry, University of Birmingham, U.K.) for his help with HPLC analysis and Prof G. Attard (University of Cardiff) for useful discussions.

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