The adherence-associated Fdp fasciclin I domain protein of the biohydrogen producer Rhodobacter sphaeroides is regulated by the global Prr pathway

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Abstract

Expression of fdp, encoding a fasciclin I domain protein important for adherence in the hydrogen-producing bacterium Rhodobacter sphaeroides, was investigated under a range of conditions to gain insights into optimization of adherence for immobilization strategies suitable for H2 production. The fdp promoter was linked to a lacZ reporter and expressed in wild type and in PRRB and PRRA mutant strains of the Prr regulatory pathway. Expression was significantly negatively regulated by Prr under all conditions of aerobiosis tested including anaerobic conditions (required for H2 production), and aerobically regardless of growth phase, growth medium complexity or composition, carbon source, heat and cold shock and dark/light conditions. Negative fdp regulation by Prr was reflected in cellular levels of translated Fdp protein. Since Prr is required directly for nitrogenase expression, we propose optimization of Fdp-based adherence in R. sphaeroides for immobilized biohydrogen production by inactivation of the PrrA binding site(s) upstream of fdp.

Keywords:
Rhodobacter sphaeroides
Biohydrogen
Green energy
Prr two-component system
Fasciclin domain
Gene regulation

Article history:
Received 23 November 2019
Received in revised form 7 July 2020
Accepted 12 July 2020
Available online xxx
Introduction

Rhodobacter sphaeroides belongs to the purple non-sulphur (PNS) group of bacteria that are widely recognized as potential ‘green energy’ producers of biohydrogen from solid food waste and food processing wastewater (reviewed in Refs. [1–4]). There are several other bacterial groups that generate hydrogen such as the bio-photolytic microalgae and cyanobacteria [5,6], and some acidogenic thermophiles and mesophiles that perform dark fermentative hydrogen production [7–9]. Examples recently reported include hydrogen-producing clostridial strains isolated from landfill leachate sludge [10], that produce high yields of up to 4.7 mol H2/mol glucose [11]; Clostridium sartagoforme and Enterobacter cloacae strains isolated from Sago industrial effluent [12]; extreme halophiles that produce biohydrogen from lignocellulose biomass in nearly saturated salt [13]; Bacillus spp. isolated from banana waste [14] and improved hydrogen production by bioaugmentation with thermophiles sampled by Thermoaerobacterium thermosaccharolyticum used to enhance thermophilic hydrogen production from corn stover hydrolysate [15]. The use of consortia of these groups of microorganisms, derived either as endogenous species isolated from biomass or from other environmental sources and used to augment the natural microbial flora has also proved a successful strategy [16–20]. However, the photofermentative processes involved in hydrogen production performed by the PNS group (represented by R. sphaeroides but also including Rhodopseudomonas capsulatus, R. palustris and Rhodospirillum rubrum), has attracted more attention because of the higher conversion efficiency and yields expected from the conversion of substrate to hydrogen and the abilities to utilise food industry wastes and solar light energy of wide ranging wavelengths (522–860 nm) [1,3,21].

R. sphaeroides has attracted particular attention, not least because of its remarkable metabolic versatility; it is able to grow phototrophically, photoheterotrophically, photoautotrophically, fermentatively and using aerobic or anaerobic respiration [21–24]. Photofermentation by PNS bacteria such as R. sphaeroides involves fermentation of organic substrates in the presence of light. Light results in the production of hydrogen and the abilities to utilise food industry wastes and solar light energy of wide ranging wavelengths (522–860 nm) [1,3,21].

There are a number of external factors reported to influence hydrogen production by R. sphaeroides, including culture medium composition (including nitrogen source and concentration, choice of organic substrate, use of mixed carbon sources and incorporation of certain metal ions), reducing agents, pH, light-dark period, illumination intensity, temperature, aerobiosis conditions and even low-intensity electromagnetic fields (e.g. Refs. [25,26] and reviewed in Refs. [2,4,9]). R. sphaeroides has been successfully used for biohydrogen production from biomass; e.g. it has recently been trialled for single-stage hydrogen production from hydrolyzed straw [27] and sugar beet molasses [28], and recently a new strain was identified for producing hydrogen using oil palm waste hydrolysate [29]. It has also been successfully used in co-culture with Enterobacter aerogenes for hydrogen production using Calophyllum inophyllum oil cake as complex carbon source [30]. Therefore, much is known about the external conditions needed to obtain and increase hydrogen production, though not all the mechanisms by which they work are yet understood.

Immobilization of PNS bacteria through biofilm formation has also been reported to be beneficial for hydrogen yields and opens up the possibilities of semi- or full-continuous culture methods for hydrogen production [1,31–33], including biophotoreactor technologies with enlarged surface areas [34–36]. Biofilm formation and adherence properties in R. sphaeroides are multifactorial, affected by flagellar location and number [37,38], chemotaxis [39], membrane cardiolipin [40], presence of functional fasciclin-1 domain protein (Fdp) [41], as well as by light-driven and other regulatory factors [42,43]. In the case of R. sphaeroides Fdp, insertionally-inactivated fdp knockout strains were reported to reduce cell adherence by 100-fold (in terms of cell number) [41]. Fdp resembles the fasciclin I (FAS1) domains found in proteins of higher organisms that have important roles in cell adhesion (Fig. 1). It also shares 60% identity (74% similarity) with the nodule-expressed Nex18 protein of Sinorhizobium meliloti [44], though there appear to be no homologues in other PNS bacteria (Fig. 1). The precise mechanism by which Fdp promotes cell adherence (a prerequisite for biofilms) in R. sphaeroides remains unknown [43]. Clearly, a deeper understanding of the factors important for establishment and maintenance of R. sphaeroides in an immobilized state will be important for improved hydrogen yields reportedly gained through immobilization, not least through employment of continuous flow photobioreactors which optimize microbial exposure to light and fresh nutrients and biomass substrates [e.g. 35]. The aim therefore of the present study was to identify conditions for Fdp expression in R. sphaeroides that promote immobilization and which can therefore ultimately be applied to hydrogen production via nitrogenase. This was investigated by testing a range of growth, chemical and physical conditions on transcriptional expression of fdp, including anaerobic conditions with reduced NH4+. We show that fdp transcription is strongly repressed by the Prr global regulatory system in wild type R. sphaeroides under all laboratory conditions tested here. This leads us to propose the future development of a new strain mutagenesis strategy for optimizing hydrogen generation based on increased attachment and biofilm development mediated by Fdp in R. sphaeroides for use in bioreactors designed for continuous biohydrogen production, through promoter engineering upstream of the fdp gene that reduces or abolishes Prr repressor binding upstream of the fdp locus.
Fig. 1 — Alignment of R. sphaeroides Fdp with fasciclin-1 proteins. Amino acid residues that are identical to Fdp are in bold and grey shading; similar amino acid residues are in bold. Secondary structural data is derived from the structure of domain pair 3 and 4 of Drosophila FAS1 and is shown below the alignment (α-helix: *****; β-strand: ≡≡≡≡). The conserved HI and H2 regions identified as protein interaction sites in several fasciclin I proteins are shown (residues 37-46 and 124-133 in Fdp, respectively).
Materials and methods

Chemicals

The chemicals used in this study were purchased from Merck (Gillingham, Dorset, UK), VWR (Lutterworth, Leicestershire, UK), or Fisher Scientific (Loughborough, Leicestershire, UK) unless otherwise stated, and were of molecular biology grade.

Bacterial strains, plasmids and growth conditions

All strains and plasmids are described in Table 1 [45–49]. E. coli strains DH5α, S17-1 and BL21[DE3] have been described previously and were routinely cultured aerobically in Luria-Bertani (LB) medium by vigorous aeration of culture vessels, or on LB agar, at 37 °C as described in Ref. [50]. Where appropriate, media were supplemented with 50 μg mL⁻¹ ampicillin and/or 50 μg mL⁻¹ kanamycin or 500 μg mL⁻¹ carbenicillin. Reporter plasmid transfer into R. sphaeroides was by conjugal transfer from E. coli S17-1 [45].

R. sphaeroides NCIB 8253 was cultured at 34 °C in M22 medium [45]; the fdp and prrA mutant strains were cultured in M22 containing 20 μg mL⁻¹ kanamycin. Liquid M22 lacked added casamino acids and contained 1.5 mM NH₄Cl which permits some nitrogenase expression under anaerobic conditions [51] but little/no hydrogen evolution anaerobically due to the absence of light and the presence of dissolved N₂. Growth was measured using culture absorbance at 680 nm (A₆₈₀). Aerobic growth of R. sphaeroides was achieved using vigorous shaking of 10 mL medium in 250 mL vessels or 500 mL in 2 L vessels. Semi-aerobic growth at 34 °C was carried out using 70 mL medium in 250 mL vessels, whilst anaerobic growth at 34 °C in the dark was achieved using M22 medium containing 60 mM dimethyl sulfoxide (DMSO).

R. sphaeroides prrA and prrB knockout mutants (PrrA and PrrB respectively) were constructed by transposon Tn5 mutagenesis of pREG464 [52,53]. Insertion sites in prrA or prrB were verified by restriction analysis and DNA sequencing. Kanamycin-resistant transconjugants were screened for loss of the suicide plasmid by Southern hybridization using parental pSUP202 as labeled probe. The correct location of the inserted transposon (and loss of intact prrA or prrB gene from the chromosome) was determined by restriction and Southern hybridization analysis. The phenotypes of the resulting strains were identical to those reported for these mutations previously [54,55], including photosynthesis- and nitrogenase-minus phenotypes, and were successfully complemented using a 4.8-kb BamH1 prr (reg) fragment described in Ref. [49].

Plasmids pBluescript-SK and pET14b have been described previously [53]. Plasmid pSUP202 is a R. sphaeroides suicide plasmid used in fdp and prr mutant construction and is the host plasmid for the R. sphaeroides genomic library and has been described previously [47]. The R. sphaeroides replicative pSDP1 reporter plasmid possessing a promoter-less lacZ gene, and pUX-Km, have both been described previously [48].

Isolation of the fdp gene has been described previously [56]. Construction of an insertional-inactivated fdp mutant was described by Ref. [41].

Reporter studies of fdp expression

The 592 bp promoter region of the fdp gene (−613 to −21 relative to the ATG start codon) was amplified by polymerase

| Table 1 – Strains and plasmids used in this study. |
|-----------------------------------------------|
| Strain/plasmid | Relevant genotype/characteristics | Source/Reference |
| R. sphaeroides NCIB 8253 | Wild type | C.N. Hunter [45] |
| PRRA | Derivative of NCIB 8253 wild type, Tn5 insertion in prrA; Km<sup>R</sup> | This work |
| PRRB | Derivative of NCIB 8253 wild type, Tn5 insertion in prrB; Km<sup>R</sup> | This work |
| Fdp | Derivative of NCIB 8253 wild type, kan insertion in fdp; Km<sup>R</sup> | Eun-Lee Jeong [41] |
| E. coli DH5-α | supE44 lacU169 Δ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | [46] |
| S17-1 | Mobilisation host. | C.N. Hunter [47] |
| BL21[DE3] | F<sup>′</sup> ompT hsdS<sub>B</sub> (r<sub>S</sub> m<sub>S</sub>) gal dcm (DE3) | Novagen |
| pSUP202 | Ap<sup>R</sup> Tc<sup>R</sup> Cm<sup>R</sup>; Mob<sup>+</sup> Tra<sup>−</sup> ColE1 replicon | C.N. Hunter [47] |
| pSDP1 | Ap<sup>R</sup> Tc<sup>R</sup> Cm<sup>R</sup>; Derivative of pK415 with promoter-less lacZ. Replicates in R. sphaeroides and E. coli | [48] |
| pSDP-FDPP | Fdp reporter; pSDP1 with fdp promoter region inserted upstream of lacZ | This work |
| pREG464 | Ap<sup>R</sup>, contains a 12 kb fragment of the R. sphaeroides prr cluster in pSUP202; prrA<sup>R</sup> prrB<sup>R</sup> prrC<sup>R</sup> | C.N. Hunter [49] |
| pUX-Km | Ap<sup>R</sup>, Km<sup>R</sup>, pUC12 with Km<sup>R</sup> gene flanked by symmetrical pUC12 multiple cloning sites | C.N. Hunter |
| pBluescript-SK | Ap<sup>R</sup>, pUC19 derivative. ColE1 ori; cloning vector with blue-white selection | Invitrogen |
| pET14b | Ap<sup>R</sup>, E. coli expression vector | Novagen |

C.N.Hunter (University of Sheffield). Km<sup>R</sup>, kanamycin resistance; Ap<sup>R</sup>, ampicillin resistance; Tc<sup>R</sup>, tetracycline resistance; Tp<sup>R</sup>, trimethoprim resistance; Sm<sup>R</sup>, streptomycin resistance.
Separation of cell proteins by two-dimensional SDS-PAGE and identification of Fdp

Protein extracts of semi-aerobically grown R. sphaeroides were prepared by batch culture to mid-exponential phase (A680 of 0.6). Cells from 300 ml cultures were harvested by centrifugation at 4 °C, and the N-terminal sequence determined by Edman degradation. The protein supernatent (soluble, cytoplasm plus periplasm) was loaded on 15% SDS-polyacrylamide resolving gels, and transferred to nitrocellulose membrane (Pall BioSupport, UK) by electroblotting for 1 h at 100 V using a Bio-Rad Mini Trans-Blot Cell. The transfer buffer contained 25 mM Tris.HCl pH 8.3, 192 mM glycine, 20% methanol, 0.025% sodium dodecyl sulphate (SDS). Membranes were washed twice for 10 min with TBS buffer (10 mM Tris.HCl pH 7.5, 150 mM NaCl) at room temperature, and incubated for 16 h in 3% (w/v) bovine serum albumin in TBS buffer. Membranes were then washed twice for 10 min each time in TBST buffer (TBS buffer containing 0.05% (v/v) Tween-20, 0.2% (v/v) Triton X-100), and then once for 10 min in TBS buffer. A 1:1000 dilution of mouse anti-RGS(H)6 monoclonal antibody (Qiagen UK) was used and the membranes incubated for 1 h at room temperature. Following two washes for 10 min each time in TBST buffer and one wash for 10 min in TBS at room temperature, a 1:5000 dilution of goat anti-mouse IgG horse radish peroxidase conjugate (Stratech Scientific Ltd, Ely, UK) in TBS buffer containing 0.05% (v/v) Tween-20, 0.2% (v/v) Triton X-100, and 0.05% sodium dodecyl sulphate (SDS). Membranes were washed twice for 10 min each time in TBST buffer and once for 10 min in TBS buffer. A 1:10000 dilution of goat anti-RGS(H)6 monoclonal antibody (Qiagen Ltd, Manchester, UK) was then prepared and incubated with the membranes for 1 h at room temperature. Following two washes for 10 min each time in TBST buffer and one wash for 10 min in TBS at room temperature, a 1:5000 dilution of goat anti-mouse IgG horse radish peroxidase conjugate (Stratech Scientific Ltd, Ely, UK) in TBS was used and the membranes incubated for 1 h at room temperature. Following four washes for 10 min each time in TBST buffer, the signal peptide region (residues 1–18), was amplified by polymerase chain reaction using upstream primer SGINT1: 5'-TCAGCCATATGAAACCGGAGACATCGTGGA -3' (NdeI cloning site underlined), and downstream primer SGEL2: 5'-GCTAG- GATCCGATCAGGCGCCGATCAGACG -3' (BamHI site underlined), using pSUP202/dp-13 as template. The 470-bp fragment was purified by gel extraction and cloned into Smal-digested pBluescript-SK to give pBFDP470. The presence of inserts with correct sequence was verified by restriction digestion analysis and sequencing. Plasmid pBFDP470 was digested with BamHI and NdeI, and the dpF fragment cloned into pET14b (NovaGen® Merck Group, UK). The final expression construct, pETfdp470, expresses a Fdp protein with a N-terminal MGSS(H)6SSGLVPRGSHM sequence followed by Fdp C-terminal MGSS(H)6SSGLVPRGSHM sequence by electroblotting for 1 h at 100 V using a Bio-Rad Mini Trans-Blot Cell. The proteins were visualised with Coomassie Brilliant Blue, excised from the membrane and the N-terminal sequence determined.

The construct was transformed into E. coli BL21 [DE3]; overexpression and purification were performed as described for RegA (PrrA) in Ref. [53].

Western blotting

To verify the presence of recombinant His-tagged Fdp purified from IPTG-induced E. coli BL21[DE3]/pET14/fdp, Western blotting was undertaken using an antibody that recognises the His6 motif as described previously [59]. Briefly, purified His6-Fdp (4 µg) was loaded on 15% SDS-polyacrylamide resolving gels. Following electrophoresis by standard methods [50], proteins were transferred to nitrocellulose membrane (Amersham Hybond-C) by electroblotting for 1 h at 100 V using a Bio-Rad Mini Trans-Blot Cell. The transfer buffer contained 25 mM Tris.HCl pH 8.3, 192 mM glycine, 20% methanol, 0.025% sodium dodecyl sulphate (SDS). Membranes were washed twice for 10 min each time in TBS buffer (10 mM Tris.HCl pH 7.5, 150 mM NaCl) at room temperature, and incubated for 16 h in 3% (w/v) bovine serum albumin in TBS buffer. Membranes were then washed twice for 10 min each time in TBST buffer (TBS buffer containing 0.05% (v/v) Tween-20, 0.2% (v/v) Triton X-100), and then once for 10 min in TBS buffer. A 1:1000 dilution of mouse anti-RGS(H)6 monoclonal antibody (Qiagen Ltd, Manchester, UK) was then prepared and incubated with the membranes for 1 h at room temperature. Following two washes for 10 min each time in TBST buffer and one wash for 10 min in TBS at room temperature, a 1:5000 dilution of goat anti-mouse IgG horse radish peroxidase conjugate (Stratech Scientific Ltd, Ely, UK) in TBS was used and the membranes incubated for 1 h at room temperature. Following four washes for 10 min each time in TBST buffer,
membranes were incubated with ECL Western blotting detection reagent (GE Healthcare, USA) and developed by autoradiography with Xograph film (Kodak Co., Herts, UK).

**Mass determinations using electrospray mass spectrometry**

Samples of purified recombinant Fdp were prepared for electrospray mass spectroscopy by the method of [60] and analysed on a single quadrupole, bench top mass spectrometer (Platform II, Micromass UK Ltd) as described by Ref. [53]. Samples were dissolved in formic acid:methanol:water (1:1:1, v/v/v) and infused into the ionisation source at a flow rate of 10 μL per minute. Data were acquired over the appropriate m/z range and were processed using the MassLynx software supplied with the instrument. The m/z spectrum was transposed onto a true molecular mass scale for more facile identification using Maximum Entropy processing techniques. An external calibration is applied, using horse heart myoglobin (MW 16,951.49 Da) as the calibrator.

**Protein determinations**

Protein content was measured using the Bio-rad DC Protein Assay Kit II (Bio-rad Laboratories Inc., Watford, Herts., UK) as outlined by the manufacturer, using bovine serum albumin as the standard.

**Results**

**Confirmation of Fdp as a member of the fasciclin I protein superfamily**

The open reading frame encoding Fdp (ORF RSP1409) was first identified as a FAS1 fasciclin I–like protein in Ref. [56] (Beta-Ig-H3/Fasciclin; https://www.uniprot.org/uniprot/Q31X26). Fdp has a predicted signal peptide at the N-terminus (residues 1–18: RKTLLALSGLGGLAAPAFA) suggesting a protein that is translocated across the inner membrane resulting in a mature 137-residue protein possessing the N-terminal sequence ETGDIVETATGA. By PSI-BLAST, the closest sequence similarity (60% identical; 74% similar) is to Sinorhizobium meliloti Nex18 (Fig. 1). It is also related (32–39% identity; 52–59% similarity) to Mycobacterium tuberculosis MPT70 and M. bovis MBP70 major secreted proteins [61], the fasciclin I domains of mammalian transforming growth factor β-induced proteins (βIG-H3 or RGD-CAP adhesion proteins, as indicated in UniProt) [62], and human osteoblast-specific factor 2 (OSF-2 or periostin) [63], which is thought to be involved in bone adhesion, and is a ligand for αvβ5 integrin [64] (Fig. 1). Drosophila FAS1 domain 4 [65,66], which is responsible for axon guidance, has 29% identity to Fdp (Fig. 1). The common feature in all these proteins, where a function is known, is their involvement in protein–protein associations. The sequence similarities are quite striking, since fasciclin I domains generally exhibit low overall sequence conservation (<20%) [66]. The two regions of high conservation recognized for the FAS1 superfamily (H1 and H2) are also strongly conserved in this putative protein. Taken together with the NMR structure of Fdp described previously [41,56], it is clear that this protein is a member of the fasciclin I protein superfamily. One unusual aspect of this particular fasciclin-domain protein is that it occurs in a free-living bacterium, and fortuitously this free-living species is well characterized regarding its physiology, metabolic versatility, molecular bases for responses to environmental change and it is also amenable to knock-out strategies. Indeed the role of Fdp in cell adhesion properties of *R. sphaeroides* has already been established; Fdp appears to promote cell adhesion as shown by insertion activation studies in which inactivation of fdp resulted in a 100-fold reduction in numbers of adherent cells in a *R. sphaeroides* adherence assay [41]. Here we investigate the regulation of expression of this adherence factor in *R. sphaeroides*, which could yield important knowledge for the establishment and continuous immobilization of bacterial cells in bioreactors.

**Transcription of Fdp is negatively regulated by the Prr signaling pathway under anaerobic and other growth conditions**

Prr is a major regulator that senses changes in external redox potential and serves as a global switch in gene expression for many genes in *R. sphaeroides* [67–69]. To investigate whether this global environment-responsive regulator controls fdp transcription, reporter studies were undertaken using the promoter region of the fdp gene linked to a lacZ reporter gene, which was expressed in both wild type and PRR mutants. Table 2 shows activity of the fdp promoter under different aerobiosis conditions, as shown by β-galactosidase measurements of *R. sphaeroides* extracts from stationary-phase cells harbouring pSDP-FDPP, a pRK415-based replicative reporter plasmid carrying 592-bp of fdp upstream sequence transcriptionally linked to lacZ. Experiments were carried out using wild type, plus two mutants PRRA and PRRB in which the prrA (encoding the response regulator PrrA) and prrB (encoding the redox sensor kinase PrrB) genes, respectively, were insertional inactivated. Anaerobic conditions were achieved using dark conditions in the presence of DMSO rather than light conditions for light harvesting, since PRR mutants are unable to grow photosynthetically. Table 2 shows that levels of fdp expression levels in aerobic and semi-aerobic cells of wild type grown on succinate-lactate medium were similar (ΔA405 units/min/mg protein = 93–100 × 10⁻³), but were slightly lower under anaerobic conditions (required for nitrogenase expression and thereby hydrogen generation) [1] (and under which the Prr pathway generates a higher level of phosphorylated PrrA, Prr-P (ΔA405 units/min/mg protein = 64 × 10⁻⁸) [70] (Table 2). Expression was significantly higher (3.7–40.3-fold) in both PRRA and PRRB strains compared with wild type under all conditions of aerobiosis in these cells grown on glucose or succinate-lactate (Table 2), demonstrating that the Prr system exerts negative control of fdp transcription under both aerobic and anaerobic nitrogenase-expressing conditions. Presumably sufficient transcriptionally-active PrrA or PrrA-P must occur for the efficient repression of the fdp promoter region observed under all aerobiosis conditions. The fold effect on expression levels in PRR mutants appears to be less marked under increasingly anaerobic conditions (though nonetheless significant), possibly suggesting that PrrA (which predominates under aerobic conditions compared with PrrA-P), is
The adherence-associated Fdp fasciclin I domain protein of the biohydrogen producer Rhodobacter sphaeroides is regulated by the global Prr pathway, International Journal of Hydrogen Energy, https://doi.org/10.1016/j.ijhydene.2020.07.108

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Table 2 – Activity of the fdp promoter in stationary phase wild type, PRRB and PRRA strains cultured under different aerobiosis conditions.

| Growth conditions          | Wild type     | PRRB          | PRRA          |
|----------------------------|---------------|---------------|---------------|
|                            | β-galactosidase (ΔA_{405}/min/mg protein × 10^5) | β-galactosidase (ΔA_{405}/min/mg protein × 10^5) | β-galactosidase (ΔA_{405}/min/mg protein × 10^5) |
|                            | Control       | pSDP-FDPP     | Control       | pSDP-FDPP     | Fold | Control       | pSDP-FDPP     | Fold |
| M22 succinate/lactate      |               |               |               |               |      |               |               |      |
| Aerobic                    | 4.2 (3.1)     | 100.0 (19.2)  | 4.9 (4.3)     | 994.9 (121.8) | 9.7  | 4.4 (7.4)     | 3487.5 (189.4) | 36.4 |
| Semi-aerobic               | 16.6 (5.8)    | 92.8 (10.1)   | 4.4 (1.6)     | 283.7 (15.5)  | 3.7  | 11.8 (1.9)    | 580.3 (14.4)  | 7.5  |
| Anaerobic                  | 16.5 (2.9)    | 64.1 (5.1)    | 6.3 (0.4)     | 252.8 (8.4)   | 5.2  | 10.1 (1.7)    | 279.3 (51)    | 5.6  |
| M22 glucose                |               |               |               |               |      |               |               |      |
| Aerobic                    | 6.1 (1.1)     | 83.9 (2.9)    | 5.4 (1.8)     | 1892.4 (18.2) | 24.3 | 9.0 (0.6)     | 3140.7 (86.6) | 40.3 |
| Semi-aerobic               | 2.2 (0.9)     | 74.7 (3.4)    | 13.5 (0.9)    | 1098.6 (2.3)  | 15.0 | 4.6 (1.4)     | 2771.5 (80.9) | 38.2 |
| Anaerobic                  | 4.4 (1.3)     | 67.7 (15.0)   | 2.0 (1.9)     | 507.8 (142.1) | 6.8  | 3.6 (0.4)     | 370.0 (103.5) | 5.0  |

The fdp promoter region was inserted upstream of lacZ as described in Methods, resulting in pSDP-FDPP. All growth experiments were conducted at 34 °C in the dark, and anaerobic growth was achieved by supplementing M22 medium with 60 mM dimethyl sulphoxide (DMSO). Growth (A_{600}) was monitored and cells harvested in late stationary phase (aerobic cultures - 30–36 h; semi-aerobic cultures - 3 days; and anaerobic DMSO-grown cultures – 9 days). β-galactosidase measurements are means derived from four separate experiments, each set performed at least in triplicate (standard deviation values in parentheses). The enzyme levels produced in corresponding pSDP1-harboring control cells are shown. Bracketed values show the standard deviation values.

a Ratio of expression levels in PRR strains compared with wild type, calculated after subtraction of control pSDP1 activity.

The overall repressor, and/or alternatively that additional aerobiosis-responsive regulators are regulating to different degrees under these conditions.

Expression of fdp was less elevated in the PRRB strain compared with PRRA under aerobic and semi-aerobic growth conditions of aerobiosis, but levels were approximately equivalent in PRRA and PRRB strains under anaerobic conditions. This suggests that whilst there is a role for PrrB in fdp regulation under all aerobiosis conditions (shown by the elevated levels of reporter in the PRRB strain), under anaerobic conditions the loss of PrrB in PRRB exerts no greater or lesser effect on fdp transcription than loss of PrrA-P in PRRA, suggesting that PrrA-P derived only from PrrB acts as the repressor under anaerobic conditions in wild type cells and/or that any additional regulators present exert their effects equally on fdp expression in anaerobically-cultured PRRA and PRRB strains (Table 2).

A similar trend was observed using glucose-containing medium, though reporter levels (and fold effects) were overall higher in aerobic and semi-aerobic mutant cells compared with those grown in the same aerobiosis conditions using succinate-lactate medium (Table 2). Under anaerobic conditions (in common with succinate-lactate), fdp expression levels were elevated 5.0–6.8 fold in the absence of a functioning Prr pathway.

Reporter studies of cells harvested at different times during batch growth revealed that in the wild type strain, under all conditions of aerobiosis including anaerobic conditions, reporter levels remained at constant low levels throughout growth (Fig. 2). By contrast, reporter levels in anaerobic/dark-grown PRRA were significantly elevated, though once again relatively similar throughout growth. Under aerobic/dark conditions (and to a lesser extent under semi-aerobic/dark conditions), reporter levels appeared more variable and possibly growth-phase dependent in the PRRB mutant. Levels in the PRRA mutant increased during lag and early exponential phase under aerobic conditions and reached a maximum level in late-exponential phase, reaching up to 99-fold those of wild type cells in the same phase of growth (Fig. 2). This may suggest the presence of additional regulators governing fdp expression, in addition to Prr. Indeed, the higher fdp expression observed in late exponential phase cells cultivated under aerobic conditions is reminiscent of gene expression control governed by quorum-based systems [71]. To perform preliminary investigations on whether quorum sensing in R. sphaeroides [72] could possibly play a role in regulation of fdp, reporter studies were undertaken using early-exponential phase aerobically-grown cells from wild type and PRRA strains and to which were added sterile culture supernatants from stationary phase wild type cells (shown to accumulate 7,8-cis-N-(tetradecenoyl) homoserine lactone, [72]) to constitute 10% of the total culture volume. Expression levels of fdp were compared to those of untreated cells after 1 h further incubation. Addition of the culture supernatant did not significantly affect expression levels; expression levels in wild type were 0.6-fold compared with untreated cells whilst in the PRRA strain levels were only 1.3-fold those of the control (Table 3). Therefore, these preliminary experiments indicate that quorum sensing plays no detectable role in fdp regulation, but further investigations should be conducted to confirm this.

Although levels of fdp expression were consistently low in wild type (compared with PRR mutants) under all conditions of aerobiosis tested (Table 2), some variation in expression levels nonetheless occurred, specifically there are significantly lower levels of expression under anaerobic conditions on succinate/lactate medium compared with aerobic and semi-aerobic conditions in the same medium (Table 2). To investigate whether other environmental factors can also affect fdp transcription in wild type, the effects of complex versus defined medium, heat versus cold shock and light versus dark conditions were investigated. For comparative purposes, strains were all cultured under aerobic conditions, so that a wider range of conditions could be investigated at a practical level. Thus, the anaerobic conditions required for nitrogenase expression were not specifically investigated.
The study also included the effects of these factors on fdp expression in PRRA strain, to determine whether any variation also occurs in the absence of the Prr pathway. The results in Table 3 demonstrate that fdp expression was sensitive to growth medium composition in both wild type and PRRA strains and maintained as described in Methods. Growth experiments were performed at 34 °C in M22 media under (a) aerobic/dark, (b) semi-aerobic/dark and (c) anaerobic/dark (in the presence of 60 mM DMSO) conditions for both wild type- and PRRA-transformed strains. Samples (1–50 ml) were taken for measurements of growth (absorbance at 680 nm, A680) (—■—) and duplicate β-galactosidase measurements (shown by the blue bars), as described in Methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2 — Activity of the fdp promoter in R. sphaeroides wild type and PRRA strains during aerobic, semi-aerobic and anaerobic batch growth. The fdp promoter region was inserted upstream of lacZ in reporter plasmid pSDP1 as described in Methods, resulting in pSDP-FDPP. A promoter-less control was also included throughout all growth experiments; reporter levels remained at the expected very low levels throughout these experiments. Plasmids were introduced into R. sphaeroides wild type and PRRA strains and maintained as described in Methods. Growth experiments were performed at 34 °C in M22 media under (a) aerobic/dark, (b) semi-aerobic/dark and (c) anaerobic/dark (in the presence of 60 mM DMSO) conditions for both wild type- and PRRA-transformed strains. Samples (1–50 ml) were taken for measurements of growth (absorbance at 680 nm, A680) (— ■ —) and duplicate β-galactosidase measurements (shown by the blue bars), as described in Methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Changes in expression levels were observed in the PRRA mutant strain. It is difficult to draw conclusions about the nature of the mechanisms by which such different regulation occurs.

To investigate possible regulatory mechanisms by which fdp is regulated under different growth conditions, studies were focused on reporter studies using pyruvate-grown cells to ascertain whether the global signaling molecule acetyl phosphate which occurs in R. sphaeroides [73] may affect fdp expression. When pyruvate is the carbon source, levels of the small phospho donor acetyl phosphate are elevated [74,75]. Acetyl phosphate is a global signaling molecule that regulates many bacterial cellular processes including nitrogen assimilation, osmoregulation, flagellar biogenesis, pilus assembly, capsule biosynthesis, biofilm development, and pathogenicity.
Table 3 – Comparison of fdp promoter activity in wild type and PRRA strains under different growth conditions.

| Treatment | Strain | Control untreated culturesa | Addition of sterile supernatentc | Foldb |
|-----------|--------|-----------------------------|---------------------------------|-------|
|           |        | Control pSDP-FDPP           | Control pSDP-FDPP               |       |
| 1         | Wild type  | 1.5 (1.5) 24.4 (7.8) 32.7 foldb | 6.5 (1.8) 21.4 (1.3) 45.1 foldb | 0.6   |
|           | PRRA    | 11.7 (3.1) 760.0 (347.7)    | 12.3 (5.8) 977.0 (58.4)         | 1.3   |
|           |         | M22 succinate lactatec       |                                 |       |
| 2         | Wild type  | 6.1 (1.0) 52.5 (6.7) 111.7 foldb | 24.5 (25.8) 165.9 (24.5)        | 3.0   |
|           | PRRA    | 4.3 (1.5) 5185.8 (297.7)    | 4.4 (0.8) 21806 (3667.8)        | 4.2   |
| 3         | Wild type  | 5.0 (5.5) 183.5 (5.6) 15.5 foldb | 1.4 1.3 (0.3) 209.3 (20.9)      | 1.2   |
|           | PRRA    | 7.3 (3.7) 2770.6 (64.3)     | 2.7 16.8 (14.8) 3868.6 (191.2)  | 1.4   |
| 4         | Wild type  | 3.2 (3.0) 141.4 (13.9) 12.7 foldb | 5.5 (1.0) 178.2 (42.4)          | 1.3   |
|           | PRRA    | 2.2 (0.0) 1802.4 (55.4)     | 16.0 (8.9) 5457.0 (24.5)        | 3.0   |

The fdp promoter region was inserted upstream of lacZ as described in Methods, resulting in pSDP-FDPP (Table 1). β-galactosidase measurements are means derived from triplicate measurements (standard deviation values in parentheses). The enzyme levels produced in corresponding pSDP1-harbouring control cells are shown.

Fold is the ratio of expression levels in the presence and absence of treatment (or Prr mutant: wildtype levels), calculated after subtraction of control pSDP1 activity. See individual treatments for more specific detail.

**Treatment 1:** Activity in early exponential phase cells in response to addition of sterile supernatent from stationary phase cells. aAerobic dark growth at 34 °C (A680) was monitored and either sterile culture supernatent from stationary phase cells were added or no addition made (Control), to early log phase cells.

**Treatment 2:** Activity in stationary phase cells cultured aerobically in rich (LB) and minimal M22 succinate-lactate media. bAerobic dark growth at 34 °C (A680) was monitored (A680) and cells harvested in stationary phase (30–36 h). Fold: ratio of expression levels in LB medium compared with those in M22 medium, calculated after subtraction of control pSDP1 activity.

**Treatment 3:** Activity in stationary phase cells following exposure to temperature shock. dAerobic growth in M22 succinate-lactate medium at 34 °C in the dark until early stationary phase before heat shock at 42 °C or cold shock at 5 °C for 4 h and cell harvesting. Fold: ratio of expression levels compared with continued standard conditions, calculated after subtraction of control pSDP1 activity.

**Treatment 4:** Activity in stationary phase cells cultured in the light or dark. eAerobic growth in M22 succinate-lactate medium at 34 °C in the dark or light was monitored (A680) and cells harvested in stationary phase (30–36 h). Fold: ratio of expression levels in the light compared with those in the same medium in the dark under aerobic conditions at 34 °C, calculated after subtraction of control pSDP1 activity. f86 W m².
One way it has been shown to exert its regulatory effects is by direct phosphorylation of response regulators of two-component systems, including *R. sphaeroides* response regulators [74,77,78]. In wild type and *fdp* mutant strains, pyruvate-grown cells consistently expressed significantly less *fdp* compared with succinate/lactate grown cells, possibly indicating greater repression by Prr, though effects due to additional regulators cannot be ruled out (Table 4). However, in the PRRA mutant strain which lacks the PrrA response regulator, the fold increase in expression levels was significantly higher (83-fold and 58-fold in semi-aerobic and anaerobic conditions, respectively) in pyruvate-grown cells compared with succinate/lactate-grown cells (10-fold) (Table 4), suggesting a possible role for acetyl phosphate and/or the presence of additional phosphorylatable regulators of *fdp* expression in addition to the Prr pathway under anaerobic (and semi-aerobic) conditions.

### Comparisons of Fdp protein levels in wild type and Prr mutants in vivo

As shown in all the reporter data described above, Fdp expression is significantly lower in the wild type strain compared with the PRR mutants. To determine whether these differences are also reflected in vivo with regard to the final translated Fdp protein, 2D SDS-PAGE analysis of cell extracts was undertaken. The Fdp protein possesses a putative signal peptide at the N-terminus, suggesting that the protein is secreted either externally or onto the cell surface, into the periplasm or is membrane-associated. Since post-translationally modified Fdp is of relatively low molecular weight, it is likely that the protein is not detected in the extracts. However, a band of similar mobility was observed in extracts of wild type and PRRA strains, and a faint band was observed in extracts of PRRB strain, indicating the presence of a protein with a similar molecular weight. These results suggest that Fdp is not secreted and is present in the periplasm or membrane. Further studies are needed to determine the exact location of Fdp in the cell.
mass (13.8 kDa), and is predicted to possess a low predicted pl (3.96) compared with other *R. sphaeroides* proteins, we reasoned that it should be possible to separate and identify the protein in two-dimensional SDS-PAGE of *R. sphaeroides* cell fractions.

Soluble extracts (including periplasmic fractions) of washed semi-aerobic wild type and PRR mutant cells were used in the 2D SDS-PAGE analysis (Fig. 3). The correct protein spot and position in the gels were identified by N-terminal sequencing (sequenced as ETGDIVETATSA, compared to the Fdp protein in the *R. sphaeroides* genome database which is ETGDIVETATGA). Characteristically, it runs anomalously in the approximate technique of SDS-PAGE, with an apparent molecular mass of 17,300 Da, higher than the predicted 13,800 Da. This is a characteristic also observed using a purified his-tagged version of Fdp expressed in *E. coli*; His_6_-Fdp possesses an apparent molecular mass of 20,100 Da in SDS-PAGE (Fig. 4) but mass spectrometry reveals a mass of 16003.9 ± 1.6 Da, in good agreement with the expected theoretical value for the recombinant protein (16,004.8 Da).

Fig. 3 shows that in PRRA and PRRB soluble extracts, levels of Fdp are significant in comparison with other cellular soluble proteins, confirming that washed cells possess abundant levels of mature, post-translationally modified Fdp. Taken together with the N-terminal sequencing data, these results demonstrate that the predicted N-terminal signal peptide is indeed cleaved in vivo, and that mature Fdp is therefore presumably exported across the inner membrane and at least into the periplasm in these strains. Fdp levels were lower or barely detectable in the wild type strain (Fig. 3), a feature consistent with the findings described above for Fdp transcription.

**Discussion**

The present study clearly demonstrates that expression of the *fdp* gene encoding a protein involved in adherence [41] is negatively regulated by the Prr global regulator in *R. sphaeroides*. Under a wide range of growth conditions tested here including different carbon sources, conditions of aerobiosis (including anaerobic conditions suitable for nitrogenase expression), rich versus defined growth media, heat/cold shock, and light versus dark conditions, elevated *fdp* promoter activity was consistently observed in PRRA and PRRB mutants compared with wild type, ranging from 3.7 to 154-fold (Tables 2–4). Increased levels of promoter activity in PRR mutants were also observed throughout batch growth under all aerobiosis conditions (Fig. 2) and whilst the highest levels of promoter activity were seen in late exponential phase cells under aerobic/dark conditions, no evidence of quorum-based regulation was found (Table 3), though further study is needed to confirm this. Interestingly Prr repression occurred under all conditions of aerobiosis, suggesting either sufficient levels of Prr-P under all these aerobiosis conditions for repression, or that unphosphorylated PrrA is also able to repress *fdp*. The regulatory activity of PrrA (or analogous RegA in other species) as well as of PrrA-P (RegA-P) has been documented previously [77,79–81].

In the absence of the Prr pathway, the increased levels of *fdp* expression varied depending on growth conditions, possibly suggesting the involvement of additional regulators involved in Fdp regulation. This was further supported by growth experiments using pyruvate as carbon source, in which elevated levels of the global signaling molecule acetyl phosphate are present; in PRR mutants growing on pyruvate, reporter levels were significantly elevated still further (Table 4), suggesting possible regulation by phosphorylatable control systems, such as two-component signal transduction systems. The effects on *fdp* expression measured using our reporter assay system were also reflected in the levels of translated Fdp protein observed in cell extracts in vivo (Fig. 3).

Evidence for the adherent properties of the wild type strain of *R. sphaeroides* used in this study has been reported previously [41]. The strain was shown here to exhibit low levels of *fdp* expression resulting in low, barely detectable levels of post-translationally modified Fdp protein in which the signal peptide has been removed in vivo (Tables 2–4, Fig. 3). Such low levels are surprising, but presumably these levels in the wild type are nonetheless sufficient to support cell adherence. There were low but detectable levels of *fdp* expression in the wild type under all conditions tested, and yet there were some limited levels of variation in these expression levels under different conditions. For example, expression levels in wild type cells cultured anaerobically in M22 succinate/lactate...
medium were 64% of those measured aerobically in the same medium (Table 2). Similarly, \textit{fdp} expression in wild type cells cultured anaerobically with pyruvate as carbon source was approximately 5-fold lower than in cells grown in the same medium semi-aerobically with succinate-lactate as carbon source (Table 4), and aerobic wild type cells cultured in rich LB medium exhibited 3-fold elevated levels of \textit{fdp} expression over cells cultured in defined M22 medium (Table 3). These variations may be due to variable regulation by Prr itself as reported for the analogous Reg pathway in \textit{R. capsulatus} which regulates in a variable way different gene sets depending on growth conditions \cite{81}. Alternatively, other Prr-independent regulatory activity may be occurring. In light of the strong regulation exerted by Prr regulation in wild type demonstrated in this study by the significantly derepressed levels of \textit{fdp} expression observed in the PRR mutants, the latter of these two possibilities appears to be the most likely explanation for the relatively low (but significant) levels of variation seen in the wild type.

The question therefore is why \textit{Fdp} expression should be subject to such strong regulation by Prr and possibly other phosphorylatable regulators under most growth conditions as demonstrated here. One possible explanation is that there may be occasions in which it is advantageous for the bacterium to experience a full reversal or partial loss of adherence ability, for example in order to enter a motile phase. Perhaps there are particular environmental conditions which occur in nature (and which were not possible to replicate in the laboratory environment), that facilitate full repression of Fdp levels equivalent to a full shut down of Fdp in the wild type, resulting in motile phase non-adherent cells. A previous study established that, in terms of cell numbers, adherence is reduced approximately 100-fold in an insertional-inactivated \textit{fdp} mutant \cite{41}. In this regard it is relevant to note that \textit{Rhodobacter} mutants in the global Prr/Reg regulatory pathway, and shown here to exhibit significantly elevated levels of Fdp in \textit{R. sphaeroides}, are defective in aerotaxis and motility \cite{81,82}. It is not suggested here that there must therefore be a direct link between elevated \textit{Fdp} levels and loss in motility and aerotaxis, as Prr is a global regulator affecting many processes, but rather that such characteristics of Prr mutants makes the above hypothesis difficult to test. Another difficulty with testing this possibility is that in the present study no laboratory conditions were identified in which Fdp levels were fully repressed in the wild type. Nonetheless, with regard to promoting permanent adherence and thereby immobilization in a bioreactor environment, we propose that engineering of the PrrA binding site upstream of the \textit{fdp} promoter to inhibit binding by the PrrA/ PrrA-P repressor may be a useful future strategy. Development of the Prr mutants themselves, which are already lacking PrrA/ PrrA-P binding and produce desirably elevated levels of Fdp, are not suitable in this case as they are defective in expression of nitrogenase for \textit{H}_2 production and other key metabolic processes such as photosynthesis and \textit{CO}_2 fixation required under light anaerobic conditions \cite{67,70,81}. Therefore, a mutagenesis strategy designed to specifically abolish or reduce PrrA binding in the \textit{fdp} promoter region and thereby ensure either strongly elevated levels of Fdp, or levels that are moderately higher than wild type levels, may be a fruitful line of future investigation.

Progress has previously been made in improving hydrogen yields through mutant analysis and genetic/metabolic engineering strategies, mainly targeting the activities of the uptake hydrogenase, poly-3-hydroxybutyric acid synthesis, nitrogenase and light harvesting systems under defined external conditions \cite{2,3,23,83-87}. Not many reports have yet appeared on mutations in transcriptional regulators; however, the studies of \cite{2,87,88} investigated the effects of HupR, HupT, NifA and NifL mutations for improving hydrogen yield in \textit{PNS} bacteria, with success in improving hydrogen production. Searches using the consensus sequence for PrrA DNA binding in \textit{R. sphaeroides} \cite{89,90} reveal two possible binding sites for PrrA to the \textit{fdp} upstream region, both of which occur in the promoter fragment used in the reporter studies described above. One starts at position −432 (5′-GGCGCGGAGATTCTGGCGG-3′). In common with sites of other repressed genes such as hydrogenase (hup), this site has a rather long half-site spacing \cite{89}. The second possible site starts at −419 (5′-GGCGCGGATGCGG-3′). Following confirmation of these PrrA binding sites, a comprehensive mutagenesis programme can be initiated.

Conclusions

Expression of the \textit{fdp} gene, which encodes an adherence factor in \textit{R. sphaeroides}, is negatively regulated by the global Prr regulatory pathway. Strains defective in either the sensor kinase PrrB or the response regulator PrrA of this pathway possess significantly elevated levels of \textit{fdp} promoter activity, which is also reflected in the levels of translated Fdp protein in \textit{R. sphaeroides} cells in vivo. One strategy to optimize or increase adherence properties of \textit{R. sphaeroides} in immobilized bioreactor applications might be to generate altered strains in which the Prr repressor activity has been reduced or removed. Mutations in the \textit{prrA} or \textit{prrB} genes themselves is not feasible, as they are required for expression of nitrogenase. We therefore propose targeted mutagenesis of the PrrA binding site upstream of the \textit{fdp} gene to reduce or remove binding by the PrrA repressor specifically at this site and thereby enhance expression of the \textit{fdp} adherence factor.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Alison Ashcroft (University of Leeds) for mass spectrometry data, Jeff Keen (University of Leeds) for the 2D SDS-PAGE, C. Neil Hunter (University of Sheffield) for provision of strains, Peter J.F. Henderson (University of Leeds, UK) and Mike Williamson (University of Sheffield) for helpful discussions and for reading the early manuscript, Samuel Kaplan
Biological Research Council [24/P13277] (to MKP-J), and a sequencing. This work was supported by the Biotechnology Moir (University of Sheffield) for protein N-terminal sions and provision of unpublished information, and Arthur Zhang K, Cao G-L, Ren N-Q. Bioaugmentation with ecosystem nitrogen and supply of plant N-fertilizer. Int J Hydrogen Energy 2014;39:6853–71.

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