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The metabolism of (*R*)-3-hydroxybutyrate is regulated by the enhancer-binding protein PA2005 and the alternative sigma factor RpoN in *Pseudomonas aeruginosa* PAO1

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A variety of soil-dwelling bacteria produce polyhydroxybutyrate (PHB), which serves as a source of energy and carbon under nutrient deprivation. Bacteria belonging to the genus *Pseudomonas* do not generally produce PHB but are capable of using the PHB degradation product (*R*)-3-hydroxybutyrate [(*R*)-3-HB] as a growth substrate. Essential to this utilization is the NAD^+-dependent dehydrogenase BdhA that converts (*R*)-3-HB into acetoacetate, a molecule that readily enters central metabolism. Apart from the numerous studies that had focused on the biochemical characterization of BdhA, there was nothing known about the assimilation of (*R*)-3-HB in *Pseudomonas*, including the genetic regulation of *bdhA* expression. This study aimed to define the regulatory factors that govern or dictate the expression of the *bdhA* gene and (*R*)-3-HB assimilation in *Pseudomonas aeruginosa* PAO1. Importantly, expression of the *bdhA* gene was found to be specifically induced by (*R*)-3-HB in a manner dependent on the alternative sigma factor RpoN and the enhancer-binding protein PA2005.This mode of regulation was essential for the utilization of (*R*)-3-HB as a sole source of energy and carbon. However, non-induced levels of *bdhA* expression were sufficient for *P. aeruginosa* PAO1 to grow on (±)-1,3-butanediol, which is catabolized through an (*R*)-3-HB intermediate. Because this is, we believe, the first report of an enhancer-binding protein that responds to (*R*)-3-HB, PA2005 was named HbcR for (*R*)-3-*h*ydroxy*b*utyrate *c*atabolism *r*egulator.

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INTRODUCTION

Bacteria of the genus *Pseudomonas* are renowned for their versatile metabolism in that they can assimilate and break down a wide assortment of compounds to meet their nutritional demands. One compound that is not often affiliated with *Pseudomonas* metabolism is (*R*)-3-hydroxybutyrate [(*R*)-3-HB]. (*R*)-3-HB is commonly recognized for its role as a ketone body produced by mammalian cells when carbohydrate availability is limiting (Akram, 2013). However, there are a number of bacteria that biosynthesize CoA derivatives of (*R*)-3-HB and other (*R*)-3-hydroxy acids, which are polymerized into macromolecular structures called polyhydroxyalkanoates (PHAs) (Anderson & Dawes, 1990; Lu *et al.*, 2009). Under starvation conditions, the PHA granule is degraded into its 3-hydroxy acid components, which can be used as sources of carbon and energy (Jendrossek & Handrick, 2002; Jendrossek *et al.*, 1996).

Pseudomonas species do not biosynthesize nor incorporate (*R*)-3-HB into their PHA reserves (Huisman *et al.*, 1989; Timm & Steinbüchel, 1990). Nonetheless, these bacteria possess an NAD^+-dependent dehydrogenase (BdhA) that converts (*R*)-3-HB into acetoacetate, thereby allowing these bacteria to use (*R*)-3-HB as a growth substrate (Feller *et al.*, 2006; Ito *et al.*, 2006; Mountassif *et al.*, 2010). BdhA dehydrogenases have been biochemically characterized for some species of *Pseudomonas*, including *P. putida* (Feller *et al.*, 2006; Paithankar *et al.*, 2007), *P. fragi* (Ito *et al.*, 2006; Nakajima *et al.*, 2005) and

Abbreviations: EMSA, electrophoretic mobility shift assay; EBP, enhancer-binding protein; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; (*R*)-2-HB, (*R*)-2-hydroxybutyrate; (*R*)-3-HB, (*R*)-3-hydroxybutyrate.

A supplementary figure is available with the online Supplementary Material.

P. aeruginosa PAO1 (Mountassif *et al.*, 2010). These studies have provided extensive information regarding catalytic properties, mechanisms and structural features of (*R*)-3-HB dehydrogenases. Despite the wealth of information regarding the biochemical properties of enzymes involved in (*R*)-3-HB metabolism, the current study is, we believe, the first to characterize the genetic regulation of *bdhA* expression and (*R*)-3-HB utilization for any given species of *Pseudomonas*.

The existence of BdhA dehydrogenases among *Pseudomonas* spp. indicates that these bacteria can assimilate (*R*)-3-HB from external sources. In an effort to understand this process, we focused on defining the genetic mechanisms surrounding (*R*)-3-HB utilization in *P. aeruginosa* PAO1. The *bdhA* (*PA2003*) gene is actually part of a bicistronic operon that starts with *PA2004*, encoding a putative transporter related to the H^+ gluconate symporter family (Winsor *et al.*, 2011). The *PA2004-bdhA* operon is preceded by a putative −24/−12 promoter recognized by the alternative sigma factor σ^{54} or RpoN (Conway & Boddy, 2012). RpoN-RNA polymerase (RNAP) holoenzymes cannot spontaneously isomerize from a closed to open complex for transcription initiation (Buck & Cannon, 1992). Additional transcriptional regulators called enhancer-binding proteins (EBPs) interact with the RpoN-RNAP holoenzyme and use the energy of nucleotide hydrolysis to mediate formation of the open complex (Morett & Segovia, 1993; Studholme & Buck, 2000). Adjacent to the *PA2004-bdhA* operon is the *PA2005* gene, which encodes a previously uncharacterized, putative EBP. The results of the current study show that RpoN and the EBP PA2005 are necessary for the induction of the *PA2004-bdhA* operon in response to (*R*)-3-HB.

METHODS

Bacteria, plasmids and media. Bacteria and plasmids used in the study are given in Table 1. The *P. aeruginosa* strains obtained from the transposon mutant library (Jacobs *et al.*, 2003) were verified using PCR as recommended by the library curators. Bacteria were grown in BD Difco Lennox broth (LB) or M63 minimal medium (Pardee *et al.*, 1959). Unless otherwise stated, bacteria were grown in 50 ml medium (in 500 ml baffled shake flasks) in a rotary shaker at 37 °C, 200 r.p.m. Medium used for growth of the *rpoN* mutant (PAO6359) of *P. aeruginosa* was supplemented with 5 mM L-Gln (Heurlier *et al.*, 2003). Plasmid selection was achieved using the following concentrations of antibiotics: kanamycin (Km) (50 μg ml^{-1} for *Escherichia coli*), carbenicillin (Cb) (100 μg ml^{-1} for *E. coli* or 200 μg ml^{-1} for *P. aeruginosa*) and gentamicin (Gm) (20 μg ml^{-1} for *E. coli* or 30 μg ml^{-1} for *P. aeruginosa*).

Standard DNA procedures. DNA was purified using Promega nucleic acid purification kits. Restriction enzymes, ligases and polymerases were products of New England BioLabs. Oligonucleotides used for PCR applications were purchased from Integrated DNA Technologies and are listed in Table 1. Genomic DNA from *P. aeruginosa* PAO1 was used for all PCR applications. PCR products were gel-purified and cloned into pCR-Blunt (Invitrogen) according to the manufacturer's instructions. Cloned DNA was verified by sequencing (Genewiz).

Cloning of the *bdhA* (*PA2003*) and *PA2004* genes. The *bdhA* and *PA2004* genes were PCR amplified using the primers BL444.f/BL444.r and BL443.f/BL443.r, respectively. The *PA2004-bdhA* operon was amplified with primers BL443.f/BL444.r. All three PCR products were individually cloned into pCR-Blunt. The *bdhA*, *PA2004* and *PA2004-bdhA* genes were then subcloned into the *Xba*I/*Sac*I sites of pBBR1MCS-5 to give pBRL496, pBRL501 and pBRL498, respectively.

Cloning of the *PA2005* ORF. The putative *PA2005* ORF was PCR amplified with the primers BL446.f/BL446.r, and the resulting PCR product was cloned into pCR-Blunt to give pBRL510. The *PA2005* ORF from pBRL510 was subcloned into the *Nde*I/*Sac*I sites of pET28b (EMD Millipore) to give pBRL516. The pBRL516 plasmid was digested with *Xba*I/*Sac*I to liberate the *PA2005* ORF with a pET-derived RBS, which was cloned into the *Xba*I/*Sac*I sites of pBBR1MCS-5 (Kovach *et al.*, 1995) to yield pJRH010. Lastly, the *PA2005* ORF from pBRL510 was cloned into the *Eco*RI site of pTrc99a (Pharmacia) with either a forward orientation (pBRL595) or reverse orientation (pBRL596) relative to the *trc* promoter.

Construction of the *PA2004-lacZ* reporter. The 1042 bp 5′ regulatory region positioned immediately upstream of the *PA2004* ORF was PCR amplified with primers BL442.f/BL442.r. This amplicon was then fused to the *lacZ* ORF of *E. coli* MG1655 with primers BL442.f/BL342.r using PCR conditions as previously described (Lundgren *et al.*, 2014). The *PA2004-lacZ* fusion PCR product was cloned into pCR-Blunt to give pBRL492. The *PA2004-lacZ* fusion in pBRL492 was cloned into the *Xba*I site of the promoterless Δ*Plac*-pBBR1MCS-5 plasmid (Lundgren *et al.*, 2014) to yield pBRL499. The GG dinucleotide of the −24 element of the RpoN promoter located 148 bp upstream of the *lacZ* ORF in pBRL499 was changed to an AA dinucleotide using QuikChange (Agilent Technologies) with the primers BL445.f/BL445.r. The resulting plasmid pBRL505 was sequenced to verify the desired mutation.

Growth studies of *P. aeruginosa* on (*R*,*S*)-3-HB. BdhA has been shown to be specific for (*R*)-3-HB (Ito *et al.*, 2006), and the BdhA of *P. aeruginosa* PAO1 was observed to synthesize acetoacetate when given (*R*,*S*)-3-HB as a substrate (Mountassif *et al.*, 2010). Therefore, the sodium salt, racemic (*R*,*S*)-3-HB (Sigma Aldrich) was used as the growth substrate, because it was more cost effective than enantiopure (*R*)-3-HB. All *P. aeruginosa* strains were grown in quadruplicate. For each replicate, M63 minimal medium that was supplemented with 30 mM (*R*,*S*)-3-HB or (±)-1,3-butanediol was inoculated to an initial OD_{600} of ∼0.1. Cultures were grown for 24 h and OD_{600} was periodically measured. For genetic complementation experiments, the medium was not supplemented with antibiotic for plasmid selection. The use of 30 mM (*R*,*S*)-3-HB or (±)-1,3-butanediol as the sole carbon source was sufficient for selection of recombinant strains.

β-Galactosidase (LacZ) assays. Each condition was tested in quadruplicate, and LacZ activity was determined using the Miller assay (Lundgren *et al.*, 2013, 2014). To monitor the change in expression of *PA2004-lacZ* over time, *P. aeruginosa* strains harbouring pBRL499 or pBRL505 were grown in LB or M63 minimal medium, which was supplemented with 30 mM sodium acetate or sodium succinate, to an OD_{600} of 0.3. Cells were then challenged with 30 mM (*R*,*S*)-3-HB, and LacZ activity was measured at 1, 2, 3 and 4 h post-induction.

The abilities of compounds to induce expression of *PA2004-lacZ* were examined by growing *P. aeruginosa* PAO1 harbouring pBRL499 in M63 minimal medium supplemented with 30 mM sodium succinate to an OD_{600} of 0.3 and then adding (*R*,*S*)-3-HB, (*R*)-3-HB, (*R*)-2-hydroxybutyrate [(*R*)-2-HB], acetoacetate, (*R*)-lactate or (*S*)-carnitine to a final concentration of 1 mM. LacZ activity was then measured 30 min post-addition of substrate. For LacZ assays

Table 1. Bacteria, plasmids and oligonucleotides used in the current study

Bacterium, plasmid or oligonucleotide	Relevant characteristics	Source
P. aeruginosa		
PAO1 M	WT	Jacobs *et al.* (2003)
PW4481	*PA2003*-H08 : : IS*phoA*/*hah* derivative of PAO1	Jacobs *et al.* (2003)
PW4483	*PA2004*-E10 : : IS*phoA*/*hah* derivative of PAO1	Jacobs *et a.* (2003)
PW4484	*PA2005*-D07 : : IS*phoA*/*hah* derivative of PAO1	Jacobs *et al.* (2003)
PAO6359	*rpoN* : : Ω-Km derivative of PAO1	Heurlier *et al.* (2003)
E. coli		
BL21(DE3)	*fhuA2* [*lon*] *ompT gal* (λ DE3) [*dcm*] Δ*hsdS*	EMD Millipore
Top10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL* (Str^R) *endA1* λ^-	Invitrogen
Plasmid		
pCR-Blunt	Cloning plasmid; Km^r	Invitrogen
pJET1.2	Cloning plasmid; Cb^r	ThermoScientific
pTrc99a	Expression plasmid; Cb^r	Pharmacia
pET28b	Expression plasmid; Km^r	EMD Millipore
pBBR1MCS-5	Broad-host strain plasmid; Gm^r	Kovach *et al.* (1995)
Δ P*lac*-pBBR1MCS-5	pBBR1MCS-5 minus *lac* promoter; Gm^r	Lundgren *et al.* (2014)
pBRL491	*bdhA* gene in pCR-Blunt; Km^r	This study
pBRL492	*PA2004-lacZ* in pCR-Blunt; Km^r	This study
pBRL494	*PA2004-bdhA* genes in pCR-Blunt; Km^r	This study
pBRL496	*bdhA* gene in pBBR1MCS-5; Gm^r	This study
pBRL498	*PA2004-bdhA* genes in pBBR1MCS-5; Gm^r	This study
pBRL499	*PA2004-lacZ* in Δ P*lac*-pBBR1MCS-5; Gm^r	This study
pBRL500	*PA2004* gene in pCR-Blunt; Km^r	This study
pBRL501	*PA2004* gene in pBBR1MCS-5; Gm^r	This study
pBRL505	*PA2004-lacZ* with mutated RpoN promoter; Gm^r	This study
pBRL510	*PA2005* ORF in pCR-Blunt; Km^r	This study
pBRL512	P_{PA2004} probe in pJET1.2; Cb^r	This study
pBRL516	*PA2005* ORF in pET28b; Km^r	This study
pBRL595	*PA2005* in pTrc99a with forwards orientation; Cb^r	This study
pBRL596	*PA2005* in pTrc99a with backwards orientation; Cb^r	This study
pJRH10	*PA2005* in pBBR1MCS-5	This study
pZS406	P_{gcvH2} probe in pJET1.2; Cb^r	This study
Oligonucleotide		
BL342.f	atgaccatgattacggattcactg	*E. coli lacZ*
BL342.r	gcagttatttttgacaccagaccaactggta	*E. coli lacZ*
BL442.f	gctggaggccgaattcttc	5′ Regulatory region of *PA2004*
BL442.r	gaatccgtaatcatggtcatcgggcatatctccacg	5′ Regulatory region of *PA2004*
BL443.f	gcatctagagccttccccatgcgaagc	*PA2004* gene
BL443.r	gcagagctctcagaccaggccggtggc	*PA2004* gene
BL444.f	gcatctagacatccagcgggagaaacagatg	*bdhA* gene
BL444.r	gcagagctcctactgcgccacccagcc	*bdhA* gene
BL445.f	cgcgattctggggcctaacacggttatcgcaacg	Mutation of RpoN promoter in 5′ regulatory region of PA2004
BL445.r	cgttgcgataaccgtgttaggccccagaatcgcg	Mutation of RpoN promoter in 5′ regulatory region of *PA2004*
BL446.f	gccatatgaacgacgccgacagcc	*PA2005* ORF
BL446.r	gcgagctctcagttcgtctctatttcgagac	*PA2005* ORF
BL447.f	gctcgattacgtctccattttg	P_{PA2004} probe
BL447.r	gaaaggcacgttgcgataacc	P_{PA2004} probe
JRH05.f	ggctcgagtttttcagcaagat	5′-Labelled Cy5 pJET1.2 primer
JRH05.r	gaatattgtaggagatcttctagaaag	5′-Labelled Cy5 pJET1.2 primer
ZS406.f	ggggctgtgccatcggctgtaacg	P_{gcvH2} probe
ZS406.r	gcggccccggccctgtcgccacgg	P_{gcvH2} probe

involving (±)-1,3-butanediol, *P. aeruginosa* strains possessing pBRL499 were grown in LB, which was supplemented with 30 mM (±)-1,3-butanediol or 1,4-butanediol. After a 16 h incubation period (37 °C, 200 r.p.m.), LacZ activity was measured for each sample.

LacZ assays involving *E. coli* were done using the *lacZ*-deficient *E. coli* strain Top10 (Invitrogen). *E. coli* Top10 was co-transformed with pBRL499 (or a *PA2004-lacZ* reporter derivative: Δ*Plac*-pBBR1MCS-5 or pBRL505) and pBRL595 (or a *PA2005* expression plasmid derivative: pTrc99a or pBRL596). Recombinant strains were grown in LB supplemented with gentamicin and carbenicillin to an OD_{600} of 0.3. Subsequently, (*R*)-3-HB, (*R*,*S*)-3-HB, 2-(*R*)-HB or acetoacetate was added to a final concentration of 30 mM, and LacZ activity was measured at 2 h post-induction.

Measurement of BdhA activity. All conditions were tested in quadruplicate. *Pseudomonas* strains were grown in LB to an OD_{600} of 0.3 and subsequently challenged with (*R*,*S*)-3-HB at a final concentration of either 0 or 30 mM. After a 4 h induction period, cells were harvested, washed and suspended in 1.0 ml lysis buffer (100 mM potassium phosphate, 300 mM NaCl, 1 μg ml^{-1} pepstatin A, 2 μg ml^{-1} leupeptin and 3 mg ml^{-1} lysozyme, pH 8.0). The cell suspensions were incubated at 25 °C for 30 min and then subjected to sonication on ice. Unlysed cells and cellular debris were removed via centrifugation. The cleared lysates were transferred to Amicon Microcon Centrifugal devices (molecular weight cut-off 10 000 Da) and washed twice with 0.5 ml lysis buffer (100 mM Tris, 300 mM NaCl, pH 8.0). Each lysate was concentrated to a final volume of 50 μl.

The Bradford assay (Pierce) was used to measure the protein concentrations of the lysates. BdhA activity was measured by the formation of NADH (extinction coefficient of 6220 M^{-1} cm^{-1}) (Brashear & Cook, 1983). For each reaction, 2 μl (~5 μg protein) lysate was added to 0.5 ml reaction buffer (100 mM Tris, 300 mM NaCl, 1.8 mM NAD^+, 25 mM (*R*)-3-HB, pH 8.0). The reaction mixture was incubated at 25 °C and the increase in absorbance at 340 nM was recorded. Each sample exhibited a linear increase in the absorbance at 340 nm for the first 8 min. BdhA activities were reported as units (U) per mg of total protein where U was defined as the formation of 1 μmol NADH min^{-1} at 25 °C, pH 8.0.

Purification and electrophoretic mobility shift assays (EMSAs) of His_6-PA2005. The *PA2005* ORF was cloned into pET28b, and the resulting plasmid pBRL516 encoded an N-terminal 6× histidine-tagged PA2005 fusion protein (His_6-PA2005). *E. coli* BL21(DE3) harbouring pBRL516 was grown in LB to an OD_{600} of ~0.6 at 37 °C with shaking (200 r.p.m.). Protein expression was induced by the addition of 0.1 mM IPTG, and the induced cultures were incubated for 12 h at 16 °C (200 r.p.m.). Cells were harvested, suspended in buffer (100 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 % v/v glycerol. 1 mg ml^{-1} lysozyme, 5 U ml^{-1} DNase I, 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} leupeptin) and then lysed by sonication on ice. Unlysed cells and debris were removed via centrifugation. The His_6-PA2005 protein was then purified from the clarified lysate using Ni-NTA Superflow resin (Qiagen). The His_6-PA2005 protein was eluted off the resin using a step elution method with elution buffer (100 mM Tris, 300 mM NaCl, pH 8.0) containing 20, 100 and 250 mM imidazole. The purified His_6-PA2005 protein was concentrated using Amicon Ultra centrifugal filter units (EMD Millipore). Protein expression and purification were monitored visually using SDS-PAGE. The concentration of purified protein was determined using the Bradford assay (Pierce).

His_6-PA2005 was expected to bind to a region preceding the RpoN promoter positioned 148 bp upstream of the *PA2004* ORF. Therefore, a 116 bp probe (P_{PA2004}), which resembled the region 237–122 bp upstream of the *PA2004* ORF, was PCR amplified with the primers BL447.f/BL447.r. For the non-specific probe, the 200 bp promoter region of *gcvH2* (P_{gcvH2}) (Lundgren *et al.*, 2013) was PCR amplified with the primers ZS406.f/ZS406.r. The P_{PA2004} and P_{gcvH2} PCR products were individually cloned into pJET1.2 (ThermoScientific) to yield the plasmids pBRL512 and pZS406, respectively. The 5′-labelled Cy5 primers JRH05.f/JRH05.r were used to PCR amplify P_{PA2004} and P_{gcvH2} from pBRL512 and pZS406, respectively. The resulting 5′-labelled Cy5 P_{PA2004} and P_{gcvH2} probes were gel-purified and used in subsequent EMSAs.

For the first set of EMSA reactions, 500 nM His_6-PA2005 was incubated with 1.0 nM 5′-labelled Cy5 P_{PA2004} probe (specific probe) or 1.0 nM 5′-labelled Cy5 P_{gcvH2} probe (non-specific probe) in EMSA buffer (25 mM Tris/acetate, 8.0 mM magnesium acetate, 10 mM KCl, 1.0 mM DTT, pH 8.0) for 30 min at 30 °C. In the second set of EMSA reactions, 1.0 nM 5′-labelled Cy5 P_{PA2004} probe was incubated with 0, 6.25, 12.5, 25, 50, 100, 200 or 400 nM PA2005 in EMSA buffer for 30 min at 30 °C. The samples were then analysed using PAGE in non-denaturing conditions and imaged using a Typhoon imager.

RESULTS

Transposon insertions into the *bdhA* (*PA2003*), *PA2004* or *PA2005* gene hindered the growth of *P. aeruginosa* PAO1 on (*R*,*S*)-3-HB

The metabolism of (*R*)-3-HB was expected to be dependent on the *PA2004-bdhA* operon, because BdhA performs an essential function in that it oxidizes (*R*)-3-HB into acetoacetate. The role of PA2004 was less clear. Because PA2004 has homology to the H^+ gluconate symporters (Winsor *et al.*, 2011), it was speculated that PA2004 might function as an (*R*)-3-HB transporter. The *PA2005* gene encodes a putative EBP, and because there is a −24/−12 or RpoN promoter 148 bp upstream of the *PA2004* ORF (Conway & Boddy, 2012), PA2005 was proposed to be the EBP that regulates expression of the *PA2004-bdhA* operon in response to (*R*)-3-HB.

Consistent with this hypothesis, a *PA2005* transposon mutant (*PA2005*::Tn) could not use (*R*,*S*)-3-HB as a sole carbon source (Fig. 1). Growth of the *PA2005*::Tn mutant on (*R*,*S*)-3-HB was restored when the *bdhA* gene, the *PA2004-bdhA* operon or the *PA2005* gene was expressed from the *lac* promoter on the broad-host-range pBBR1MCS-5 plasmid (Fig. 1a). This finding suggested that the diminished capacity of the *PA2005*::Tn mutant to grow on (*R*,*S*)-3-HB might be a result of insufficient expression of the *bdhA* gene. Not surprisingly, a *bdhA* transposon mutant (*bdhA*::Tn) (Fig. 1b) and a *PA2004*::Tn mutant (Fig. 1c) also failed to utilize (*R*,*S*)-3-HB as a carbon source.

Expression of a *PA2004-lacZ* construct was induced by (*R*)-3-HB

The expression of the *PA2004-bdhA* operon was expected to be regulated by PA2005 in response to (*R*)-3-HB availability. Therefore, it was first determined if the *PA2004-bdhA* operon was in fact inducible by (*R*)-3-HB.

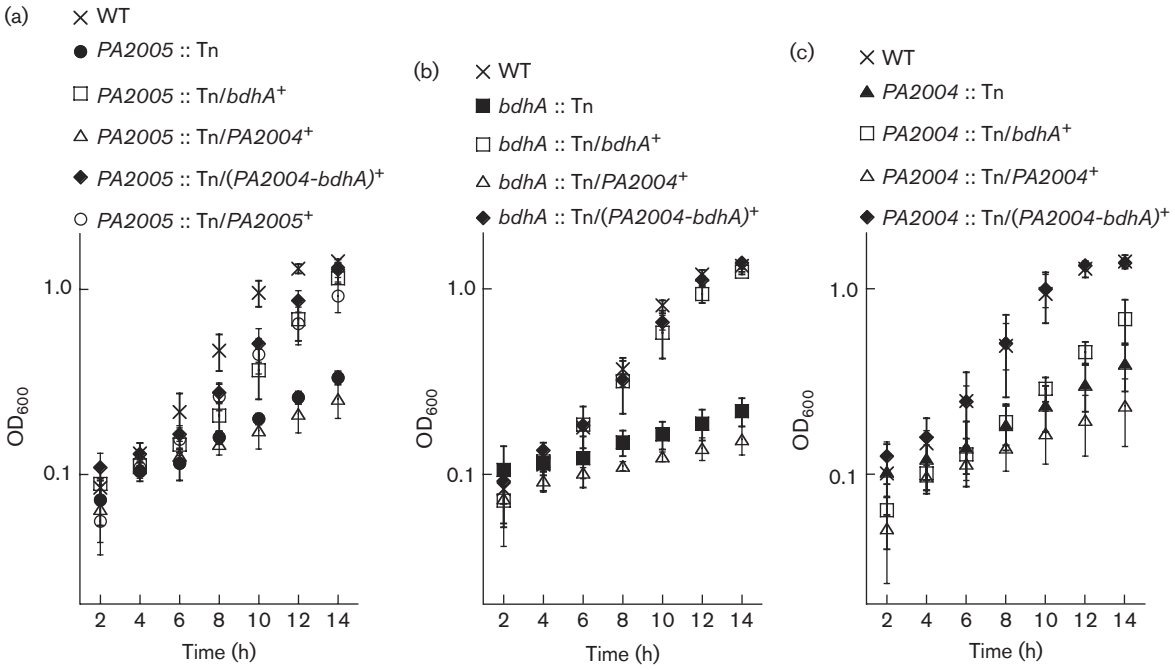

Fig. 1. Transposon (Tn) insertions in the *bdhA*, *PA2004* and *PA2005* genes negatively affected the utilization of (*R*,*S*)-3-HB in *P. aeruginosa* PAO1.Growth deficiencies were observed for (a) *PA2005* : : Tn, (b) *bdhA* : : Tn and (c) *PA2004* : : Tn mutants of *P. aeruginosa* PAO1 on 30 mM (*R*,*S*)-3-HB. Expression of *bdhA* (*bdhA* $^{+}$), the *PA2004-bdhA* operon [(*PA2004-bdhA*)$^{+}$] or *PA2005* (*PA2005* $^{+}$) from the *lac* promoter of the pBBR1MCS-5 plasmid restored growth of the *PA2005* : : Tn mutant on (*R*,*S*)-3-HB. The *bdhA* : : Tn and *PA2004* : : Tn mutants were also complemented with plasmid-derived expression of *bdhA* (*bdhA* $^{+}$) or the *PA2004-bdhA* operon [(*PA2004-bdhA*)$^{+}$]. Data points represent mean values ± SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (α-value of 0.05) to identify significant changes ($P < 0.0001$).

To achieve this goal, the 5′ regulatory region (1042 bp) located immediately upstream of the *PA2004* start codon was fused to the *lacZ* ORF of *E. coli* MG1665, and the resulting *PA2004-lacZ* fusion was cloned into the promoterless plasmid ΔP*lac*-pBBR1MCS-5 (Lundgren *et al.*, 2014). *P. aeruginosa* PAO1 harbouring the *PA2004-lacZ* reporter was grown in M63 minimal medium supplemented with 30 mM succinate. When the cells reached an OD_{600} of 0.3, they were challenged with various substrates added at a final concentration of 1 mM. Within 30 min of the addition of either (*R*)-3-HB or (*R*,*S*)-3-HB, *PA2004-lacZ* expression increased twofold (Fig. 2a). Other tested substrates, including (*R*)-2-HB, butyrate, acetoacetate, (*R*)-lactate and (*S*)-carnitine, did not induce expression of *PA2004-lacZ* (Fig. 2b). Increased expression of *PA2004-lacZ* was specific to (*R*)-3-HB, indicating that this molecule is an inducer of the *PA2004-bdhA* operon in *P. aeruginosa* PAO1.

RpoN was required for the induction of *PA2004-lacZ* by (*R*)-3-HB

There is a putative RpoN promoter located 148 bp upstream of the *PA2004* ORF (Conway & Boddy, 2012). This RpoN promoter, TGGCACGGTTATCGCA, has the highly conserved 'GG' and 'GC' (underlined) nucleotides positioned at the −24 and −12 elements, respectively (Barrios *et al.*, 1999). Since one of the hallmark attributes of RpoN is its role in the assimilation of various organic compounds, RpoN was considered to regulate expression of the *PA2004-bdhA* operon in response to (*R*)-3-HB. In support of this hypothesis, expression levels of the *PA2004-lacZ* reporter were fourfold lower in an *rpoN* mutant compared with WT *P. aeruginosa* PAO1 challenged with 30 mM (*R*,*S*)-3-HB (Fig. 3). In parallel, the conserved 'GG' nucleotides of the −24 element were changed to 'AA' in the RpoN promoter of the *PA2004-lacZ* reporter. This 'AA' substitution at the −24 element reduced the expression of *PA2004-lacZ* by more than fourfold (Fig. 3). Collectively, RpoN and its cognate −24/−12 promoter were necessary for (*R*)-3-HB induction of *PA2004-lacZ*, increasing it from a background (non-induced) level of 200 MU to >800 MU.

Induction of the *PA2004-lacZ* reporter was dependent on *PA2005*

(*R*)-3-HB and RpoN were observed to be crucial determinants for the induction of the *PA2004-lacZ* reporter in *P. aeruginosa* PAO1. These results in combination with the previous finding

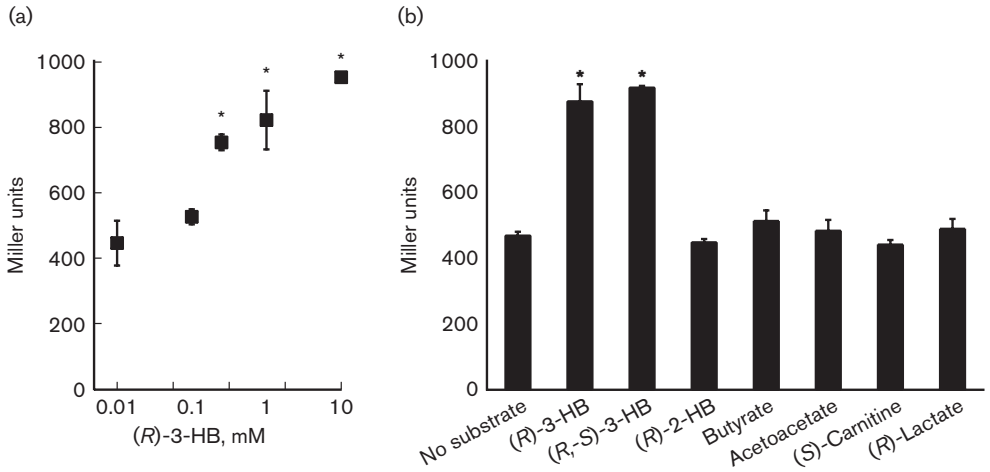

Fig. 2. (*R*)-3-HB induced expression of a *PA2004-lacZ* reporter in *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 harbouring a *PA2004-lacZ* reporter was grown to an OD_{600} of 0.3 in M63 minimal medium supplemented with 30 mM succinate and subsequently challenged with (a) various concentrations of (*R*)-3-HB or (b) various substrates provided at final concentrations of 1.0 mM. LacZ activity was measured 30 min post-addition of substrate. As shown, racemic and enantiopure (*R*)-3-HB were the only compounds that induced expression of the *PA2004-lacZ* reporter (twofold increase in LacZ activity). Notably, a concentration of 500 μM (*R*)-3-HB was found to be sufficient to induce expression of *PA2004-lacZ*. Data points represent mean values ± SD ($n=4$). ANOVA was performed using a Dunnett's post-hoc test (α-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked with an asterisk.

that a *PA2005*::Tn mutant had reduced growth on (*R*)-3-HB made PA2005 a prime candidate for being the EBP that participates with RpoN to activate transcription of the *PA2004-bdhA* operon in response to (*R*)-3-HB. Indeed, the addition of 30 mM (*R*,*S*)-3-HB did not induce expression of *PA2004-lacZ* in a *PA2005*::Tn mutant (Fig. 4). For *bdhA*::Tn, *PA2004*::Tn and WT *P. aeruginosa* PAO1, *PA2004-lacZ* expression increased more than threefold with the addition of 30 mM (*R*,*S*)-3-HB (Fig. 4).

The LacZ findings were validated by assaying (*R*)-3-HB dehydrogenase activity for the *bdhA*::Tn, *PA2004*::Tn and *PA2005*::Tn mutants. Cells were grown in LB to an OD_{600} of 0.3 and subsequently induced with either 0 or 30 mM (*R*,*S*)-3-HB. At 4 h post-induction, cells were harvested, washed, and lysed by sonication, and the resulting lysates were assayed for BdhA activity. For WT cells, BdhA activity increased from 93 (±5.9) to 395 (±17) U mg^{-1} with the addition of (*R*,*S*)-3-HB. There was no detectable BdhA activity present in the *bdhA*::Tn mutant under either condition. The *PA2004*::Tn mutants had a non-induced BdhA activity of 16 (±0.7) U mg^{-1}, which marginally increased to 23 (±2.4) U mg^{-1} with the addition of (*R*,*S*)-3-HB. For the *PA2005*::Tn mutant, non-induced and induced cells had BdhA activities of 23 (±0.9) and 19 (±2.3) U mg^{-1}, respectively. These values were ~20-fold lower than that of WT *P. aeruginosa* PAO1 challenged with (*R*,*S*)-3-HB. The measured BdhA activities are consistent with the LacZ data, indicating that *PA2005* is necessary for the induction of BdhA expression in response to (*R*)-3-HB.

(*R*)-3-HB induced expression of *PA2004-lacZ* in *E. coli* Top10 that heterologously expressed *PA2005*

Expression of the *PA2004-lacZ* reporter was assayed in non-native *E. coli* Top10, which simultaneously expressed the *PA2005* gene from pTrc99a. As shown in Fig. 5, the basal expression of *PA2005* from the *trc* promoter of pTrc99a caused a >60-fold induction of *PA2004-lacZ* in *E. coli* Top10 when challenged with either (*R*)-3-HB or (*R*,*S*)-3-HB. LacZ activity increased from 50 MU to >3000 MU at 2 h post-addition of 30 mM (*R*)-3-HB or (*R*,*S*)-3-HB. (*R*)-3-HB induction was not observed in the absence of *PA2005* or when the RpoN promoter in the *PA2004-lacZ* reporter was altered (Fig. 5a). As observed earlier, the addition of (*R*)-2-HB or acetoacetate did not induce expression of *PA2004-lacZ* (Fig. 5b). These findings demonstrate that, even in a non-native host, RpoN and PA2005 are sufficient and essential for the (*R*)-3-HB induction of the *PA2004-lacZ* reporter, and thus are key regulators of the *PA2004-bdhA* operon in *P. aeruginosa* PAO1.

Promoter region of *PA2004* was bound by purified His_6-PA2005

EMSA was used to determine if PA2005 actually binds to the *PA2004* promoter region. To this end, a 5′-labelled Cy5 probe, which spanned the region 237–122 bp upstream of the *PA2004* ORF, was used in an EMSA with purified His_6-PA2005. As shown in Fig. 6a, purified His_6-PA2005 retarded the mobility of the *PA2004* promoter probe but

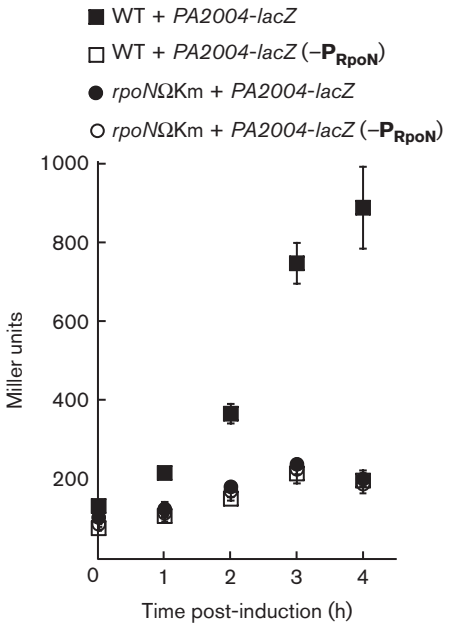

Fig. 3. (*R*)-3-HB induction of *PA2004-lacZ* was RpoN dependent. There is a putative −24/−12 or RpoN promoter located 148 bp upstream of the *PA2004* ORF, suggesting that RpoN might be involved in the transcription of the *PA2004-bdhA* operon in response to (*R*)-3-HB. Addition of 30 mM (*R*,*S*)-3-HB did not induce expression of the *PA2004-lacZ* reporter in an *rpoN* mutant (*rpoN*ΩKm) of *P. aeruginosa* PAO1. Furthermore, substitution of the conserved 'GG' nucleotides of the −24 element with 'AA' in the RpoN promoter (−P_{RpoN}) of the *PA2004-lacZ* reporter made it unresponsive to 30 mM (*R*,*S*)-3-HB. Cells were grown in LB supplemented with 5 mM L-Gln to an OD_{600} of 0.3 and then challenged with 30 mM (*R*,*S*)-3-HB. LacZ activity was determined 1, 2, 3 and 4 h post-induction. L-Gln was provided to support the growth of the *rpoN* mutant (Heurlier *et al.*, 2003). Data points represent mean values ± SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (α-value of 0.05) to identify significant changes ($P < 0.0001$).

did not change the mobility of a non-specific target promoter, i.e. the promoter region of *gcvH2*, which encodes a glycine cleavage protein (Lundgren *et al.*, 2013). When increasing concentrations of His_6-PA2005 from 0 to 400 nM were incubated with the *PA2004* promoter probe, a mobility shift was observed for His_6-PA2005 concentrations as low as 12.5 nM and the shift intensity increased with the concentration of His_6-PA2005 (Fig. 6b). These results indicate that PA2005 binds with high specificity and affinity to the *PA2004* promoter region.

Assimilation of (±)-1,3-butanediol requires the *bdhA* gene

(*R*)-3-HB utilization was dependent on the *bdhA* gene and its regulator PA2005. However, it was not known whether BdhA and/or PA2005 were necessarily essential for the assimilation of other compounds that are metabolized through (*R*)-3-HB intermediates. The compound (±)-1,3-butanediol is a common industrial chemical that is converted into (*R*)-3-HB by both eukaryotic and bacterial cells (Kersters & De Ley, 1963; Tate *et al.*, 1971; Ugwu *et al.*, 2011). Nothing is known about the metabolism of (±)-1,3-butanediol in *Pseudomonas* except for a study that identified this molecule as a substrate for a quinoprotein alcohol dehydrogenase of *P. putida* (Toyama *et al.*, 1995). We found that *P. aeruginosa* PAO1 could grow on (±)-1,3-butanediol when provided as a carbon source (Fig. 7). This metabolism was dependent on *bdhA*, as the *bdhA*::Tn mutant was unable to grow on (±)-1,3-butanediol (Fig. 7a). Notably, this result reaffirms that (*R*)-3-HB is an intermediate of (±)-1,3-butanediol metabolism in *P. aeruginosa* PAO1. Disruptions of the *PA2004* and *PA2005* genes did not abolish growth of *P. aeruginosa* PAO1 on (±)-1,3-butanediol. The *PA2004*::Tn (Fig. 7b) and *PA2005*::Tn (Fig. 7c) mutants exhibited only reduced growth on this diol.

Expression of the *PA2004-lacZ* reporter remained unchanged (background levels) for *PA2004*::Tn, *PA2005*::Tn and WT *P. aeruginosa* PAO1 cells when grown on (±)-1,3-butanediol (Fig. S1, available in the online Supplementary Material). In contrast, expression of *PA2004-lacZ* increased twofold for the *bdhA*::Tn mutant in the presence of (±)-1,3-butanediol. Only in the absence of *bdhA* does intermediate (*R*)-3-HB reach concentrations that induce expression of the *PA2004-lacZ* reporter. It would appear that the catabolism of (±)-1,3-butanediol does not generate sufficient levels of (*R*)-3-HB to trigger the induction of the *PA2004-bdhA* operon via RpoN-PA2005.

DISCUSSION

The *PA2004-bdhA* operon is conserved among many *Pseudomonas* spp., suggesting that it is a core unit and regulatory site in (*R*)-3-HB catabolism. Analysis of a *PA2004-lacZ* reporter and BdhA enzymic activity identified several key factors involved in the expression of the *PA2004-bdhA* operon in *P. aeruginosa* PAO1. First, the expression of the *PA2004-bdhA* operon is inducible by (*R*)-3-HB (Fig. 2). This induction or transcriptional activation was completely dependent on the alternative sigma factor RpoN and its cognate −24/−12 promoter positioned 148 bp upstream from the start codon of *PA2004*. (*R*)-3-HB did not induce expression of the *PA2004-lacZ* reporter in an *rpoN* mutant, while replacement of the conserved 'GG' motif of the −24 element with 'AA' in the RpoN promoter of the *PA2004-lacZ* reporter rendered it unresponsive to exogenous (*R*)-3-HB (Fig. 3). Interestingly, the operons encoding the BdhA-PA2004 homologues in *P. putida* KT2440 (*PP_3073-PP_3074*) and *Pseudomonas fluorescens* SBW25 (*PFLU2628-PFLU2629*) are preceded by RpoN promoters at 65 and 72 bp upstream of *PP_3074* and *PFLU2629*, respectively (Conway & Boddy, 2012).

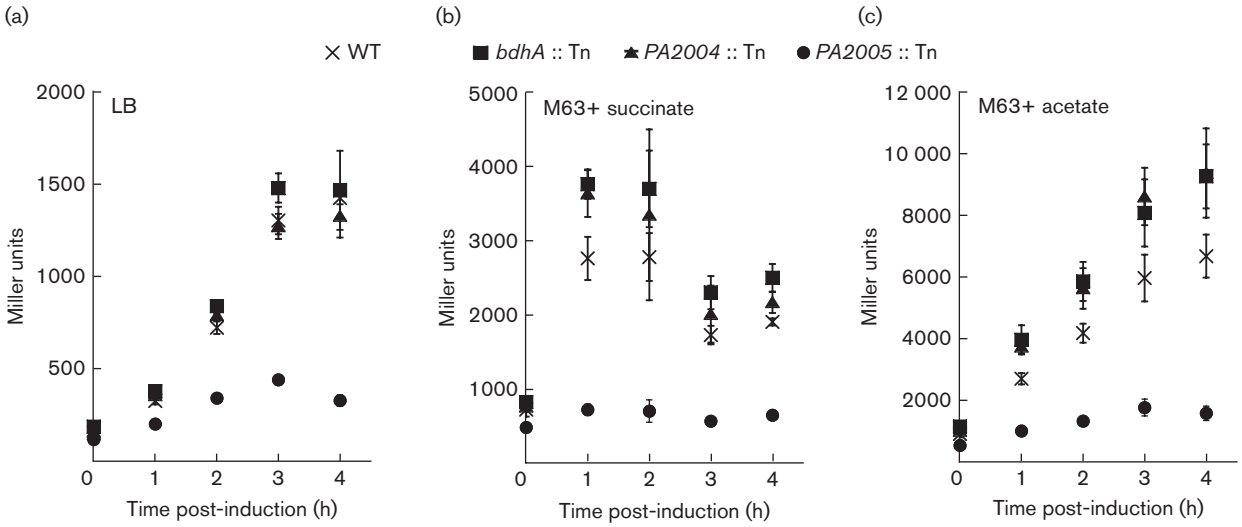

Fig. 4. (*R*)-3-HB induction of *PA2004-lacZ* required the *PA2005* gene. *bdhA* : : Tn, *PA2004* : : Tn, *PA2005* : : Tn and WT *P. aeruginosa* PAO1 harbouring the *PA2004-lacZ* reporter were grown in (a) LB or M63 minimal medium that was supplemented with either (b) 30 mM succinate or (c) 30 mM acetate. At an OD_{600} of 0.3, (*R*,*S*)-3-HB was added to a final concentration of 30 mM, and LacZ activity was determined 1, 2, 3 and 4 h post-induction. LacZ activity increased for *bdhA* : : Tn, *PA2004* : : Tn and WT cells when challenged with (*R*,*S*)-3-HB. In contrast, (*R*,*S*)-3-HB did not induce LacZ activity in the *PA2005* : : Tn mutant under any growth condition. Data points represent mean values ± SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (α-value of 0.05) to identify significant changes (P<0.0001).

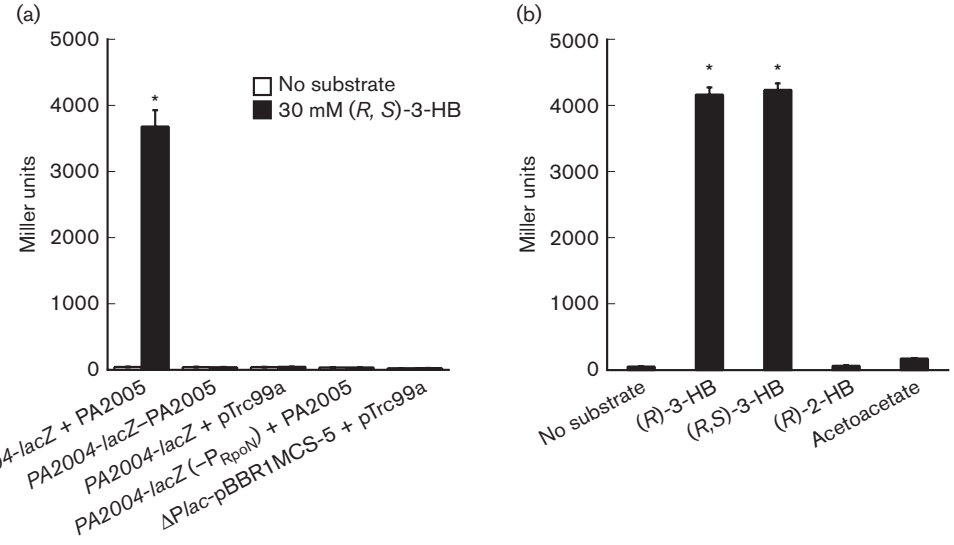

Fig. 5. PA2005 was essential for (*R*)-3-HB induction of *PA2004-lacZ* in *E. coli*. The *lacZ*-deficient *E. coli* Top10 strain was co-transformed with the *PA2004-lacZ* reporter and the *PA2005* gene, which was carried on the pTrc99a expression plasmid. (a) Basal expression of the *PA2005* gene from the *trc* promoter of pTrc99a was sufficient for (*R*)-3-HB induction of *PA2004-lacZ* (*PA2004-lacZ* + PA2005). Induction was not observed for the *PA2004-lacZ* reporter when (i) the *PA2005* gene was cloned in a backwards orientation relative to the *trc* promoter in pTrc99a (*PA2004-lacZ* – PA2005), (ii) no *PA2005* gene was present, only pTrc99a plasmid (*PA2004-lacZ* + pTrc99a), or (iii) the 'GG' nucleotides of the −24 element of the RpoN promoter were substituted with 'AA' [*PA2004-lacZ* (–P_{RpoN}) + PA2005]. (b) Induction of *PA2004-lacZ* was specific for (*R*)-3-HB. In total, these results argue that PA2005 is the EBP responsible for activating transcription from the RpoN promoter upstream of *PA2004-bdhA* in response to (*R*)-3-HB. Cells were grown in LB to an OD_{600} of 0.3 and then challenged with 30 mM of substrate. LacZ activity was measured 2 h post-induction. Data points represent mean values ± SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (α-value of 0.05) to identify significant changes (P<0.0001), which are marked with an asterisk.

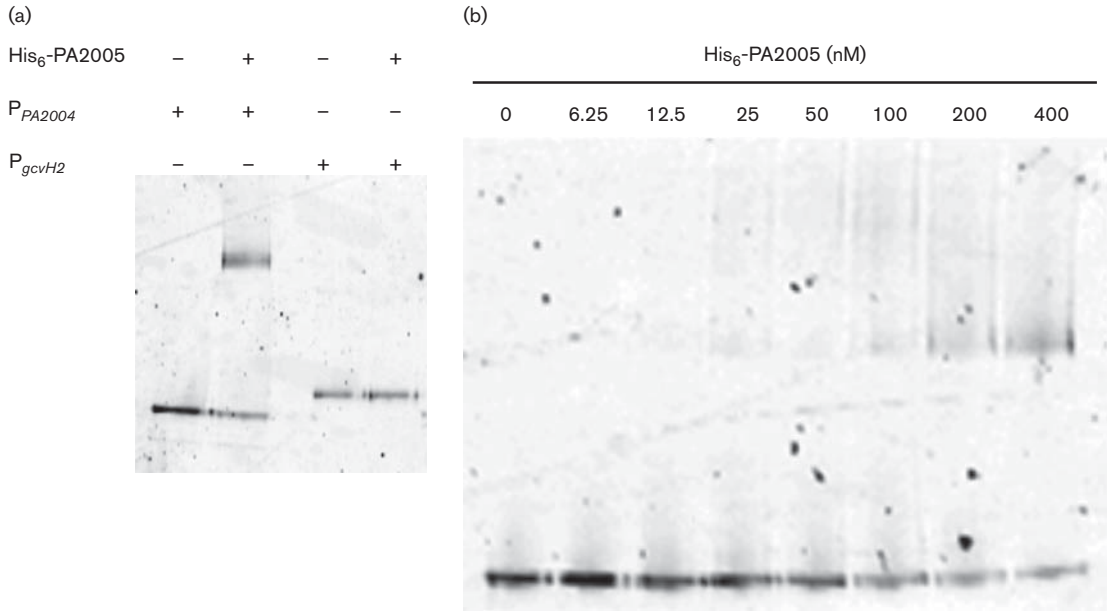

Fig. 6. The promoter region of *PA2004* was bound by His_6-PA2005.EMSAs were performed with His_6-PA2005 and 1.0 nM 5′-labelled Cy5 probe DNA unless specified otherwise. (a) P_{PA2004} (specific) or P_{gcvH2} (non-specific) were incubated in the absence (lanes 1 and 3, respectively) or presence (lanes 2 and 4, respectively) of 500 nM PA2005.The shift in the position of P_{PA2004} in lane 2 confirms that PA2005 binds to P_{PA2004}. No shift corresponding to binding of the non-specific probe P_{gcvH2} by PA2005 was observed in lane 4. (b) His_6-PA2005 (0 to 400 nM) was incubated with P_{PA2004}. A shift in P_{PA2004} was observed for His_6-PA2005 concentrations as low as 12.5 nM, indicating that His_6-PA2005 binds with high affinity to P_{PA2004}. The intensity of the shift increased with increasing His_6-PA2005 concentration.

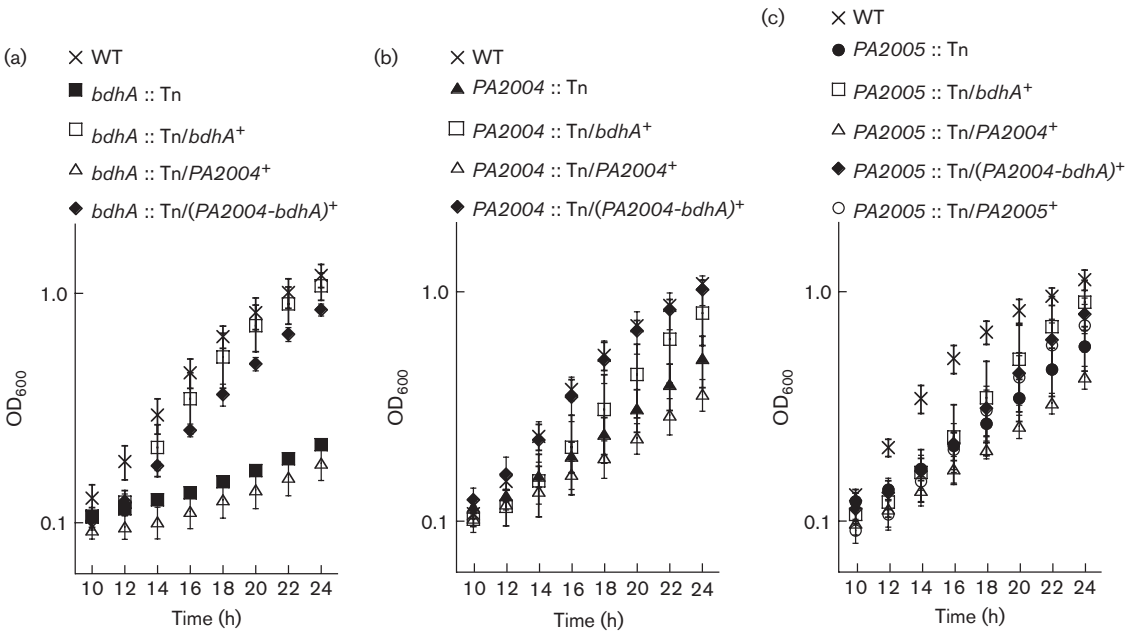

Fig. 7. The *bdhA* gene was necessary for the growth of *P. aeruginosa* PAO1 on (±)-1,3-butanediol. (*R*)-3-HB is an intermediate of (±)-1,3-butanediol catabolism. (a) The *bdhA* : : Tn mutant failed to grow on (±)-1,3-butanediol as the sole carbon source. (b, c) The *PA2004* : : Tn (b) and *PA2005* : : Tn (c) mutants displayed reduced growth on (±)-1,3-butanediol, which suggested that the expression levels of the *bdhA* gene in these mutants were high enough to produce adequate amounts of (*R*)-3-HB dehydrogenase for converting intermediate (*R*)-3-HB into acetoacetate. Data points represent mean values±SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (α-value of 0.05) to identify significant changes ($P<0.0001$).

Additionally, adjacent to each of these operons is a gene (*PP_3075*, *PFLU2630*) encoding an EBP that is homologous to PA2005. EBPs are essential participants in RpoN-mediated transcription (Studholme & Buck, 2000), and therefore regulation of (*R*)-3-HB assimilation for some *Pseudomonas* spp. might be dependent on RpoN and an EBP homologous to PA2005.

The results of this study strongly suggest that the *PA2005* gene encodes the EBP that interacts with RpoN to activate transcription of the *PA2004-bdhA* operon in response to (*R*)-3-HB. It was observed that the addition of exogenous (*R*)-3-HB did not induce expression of the *PA2004-lacZ* reporter nor did it lead to an increase in BdhA activity in a *PA2005*::Tn mutant compared with WT *P. aeruginosa* PAO1 (Fig. 4). We also found that (*R*)-3-HB induced the expression of the *PA2004-lacZ* reporter in *E. coli*, but only if the *E. coli* cells were expressing the *PA2005* gene from the *trc* promoter of the plasmid pTrc99a (Fig. 5). The presence of PA2005 was essential for the (*R*)-3-HB induction of *PA2004-lacZ* in non-native *E. coli*. Lastly, purified PA2005 protein did bind to a probe resembling the promoter region of *PA2004* (Fig. 6), which supports PA2005 being a direct regulator of the *PA2004-bdhA* operon.

The EBP PA2005 is not a homologue of any previously characterized EBPs. Pfam analysis of PA2005 identified an N-terminal PAS-4 domain (residues 25–116), the central RpoN-interaction domain (residues 158–326) and a C-terminal FIS-type helix–turn–helix (HTH) (residues 425–466). The PAS-4 domain most likely has a role in sensing intracellular (*R*)-3-HB, whereas the FIS-type HTH is expected to bind to a DNA motif upstream of the RpoN promoter of *PA2004-bdhA*. More in-depth EMSAs with PA2005 are expected to identify the DNA-binding sites for this transcriptional regulator. Based on the results in the current study, PA2005 was given the name HbcR for (*R*)-3- hydroxybutyrate catabolism regulator.

The assimilation of (*R*)-3-HB as a main carbon source was dependent on the induction of the *PA2004-bdhA* operon via RpoN-PA2005. In contrast, induction of the *PA2004-bdhA* operon was not observed when *P. aeruginosa* PAO1 was grown on (±)-1,3-butanediol even though (*R*)-3-HB is an intermediate in the breakdown of this diol (Fig. S1). This finding indicates that non-induced levels of BdhA are sufficient to convert any available (*R*)-3-HB into acetoacetate during the metabolism of (±)-1,3-butanediol. Importantly, non-induced levels of BdhA might support the growth of *P. aeruginosa* PAO1 on other molecules in which the formation of (*R*)-3-HB is a rate-limiting step in the catabolic process. For example, *Pseudomonas* spp. have been reported to hydrolyse PHB polyhydroxybutyrate (PHB) granules produced by other bacteria (Jendrossek *et al.*, 1996), and subsequently use the liberated or free (*R*)-3-HB as a growth substrate. If the hydrolysis of the PHB granule is relatively slow then (*R*)-3-HB is not expected to accumulate to concentrations that lead to the induction of the *PA2004-bdhA* operon, and thus a similar situation to that described for (±)-1,3-butanediol metabolism will be observed. Whether *P. aeruginosa* PAO1 can degrade PHB granules remains to be determined, and what implications (*R*)-3-HB induction of *PA2004-bdhA* has for the assimilation of PHB granules in the environment is a subject worth exploring.

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