Transcriptional activation of the *Azotobacter vinelandii* polyhydroxybutyrate biosynthetic genes *phbBAC* by PhbR and RpoS

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We previously showed that in *Azotobacter vinelandii*, accumulation of polyhydroxybutyrate (PHB) occurs mainly during the stationary phase, and that a mutation in *phbR*, encoding a transcriptional regulator of the AraC family, reduces PHB accumulation. In this study, we characterized the roles of PhbR and RpoS, a central regulator during stationary phase in bacteria, in the regulation of expression of the PHB biosynthetic operon *phbBAC* and *phbR*. We showed that inactivation of *rpoS* reduced PHB accumulation, similar to the *phbR* mutation, and inactivation of both *rpoS* and *phbR* resulted in an inability to produce PHB. We carried out expression studies with the wild-type, and the *rpoS*, *phbR* and double *rpoS-phbR* mutant strains, using quantitative RT-PCR, as well as *phbB::gusA* and *phbR::gusA* gene fusions. These studies showed that both PhbR and RpoS act as activators of *phbB* and *phbR*, and revealed a role for PhbR as an autoactivator. We also demonstrated that PhbR binds specifically to two almost identical 18 bp sites, TGTCACCAA-N₄-CACTA and TGTCACCAA-N₄-CAGTA, present in the *phbB* promoter region. The activation of *phbB* and *phbR* transcription by RpoS reported here is in agreement with the observation that accumulation of PHB in *A. vinelandii* occurs mainly during the stationary phase.

Received 3 June 2011

Revised 13 July 2011

Accepted 18 July 2011

INTRODUCTION

Azotobacter vinelandii is a soil bacterium that undergoes differentiation to form cysts resistant to desiccation, and produces the polyester polyhydroxybutyrate (PHB), an industrially important polymer used as a biodegradable plastic. The sigma factor RpoS is a central regulator during the stationary phase in bacteria (for a recent review, see Navarro Llorens *et al.*, 2010). In *A. vinelandii*, RpoS is involved in the formation of mature cysts (Cocotl-Yañez *et al.*, 2011), and is also required for survival under oxidative stress and under conditions of either carbon or nitrogen starvation (Sandercock & Page, 2008). Synthesis of PHB in *A. vinelandii* occurs entering the stationary phase (Martínez *et al.*, 1997), and is carried out in three enzymic steps: a β -ketothiolase catalyses the condensation of two molecules of acetyl-CoA to produce acetoacetyl-CoA, which is reduced by the NADPH-dependent acetoacetyl-CoA reductase to form β -hydroxybutyryl-CoA, which is polymerized by the PHB synthase. The genes encoding the β -ketothiolase *phbA*, acetoacetyl-CoA reductase *phbB* and PHB synthase *phbC* are organized in the *phbBAC* operon (Segura *et al.*, 2003). The *phbR* gene,

located upstream and in the opposite direction to *phbB*, encodes a transcriptional activator of the AraC family, and a mutation in *phbR* reduces accumulation of PHB (Peralta-Gil *et al.*, 2002). Transcription of the *phbBAC* gene cluster has been shown to start from two overlapping promoters, p_{B1} and p_{B2}, and a reduction in the transcription of *phbB* from its p_{B1} promoter in the *phbR* mutant has been revealed in an S1-mapping experiment (Peralta-Gil *et al.*, 2002). A consensus sequence recognized by RpoS is present in p_{B2} (Peralta-Gil *et al.*, 2002). Two start sites for the transcription of *phbR* have also been identified, defining the p_{R1} and p_{R2} promoters. The binding targets for some regulators of the AraC family, such as MelR from *Escherichia coli*, consist of repeated sequences 18 nt long (Belyaeva *et al.*, 2000). Two almost identical 18 bp sequences present in the *phbB* regulatory region, designated R1 and R2, have been identified. Site R1 overlaps the –35 region of p_{B1}, and R2 is located 3 nt upstream of R1. Thus, R1 and R2 have been proposed to be PhbR-binding sites. Four other less conserved 18 bp sequences, designated R3, R4, R5 and R6, have also been identified within the intergenic *phbR-phbB* region (Peralta-Gil *et al.*, 2002).

In this study we show that synthesis of PHB, as well as transcription of *phbB* and *phbR*, is abrogated in the absence of both PhbR and RpoS, and that the PhbR protein binds specifically to the R1 and R2 sequences present in the *phbB*

Abbreviations: EMSA, electrophoretic mobility shift assay; MBP, maltose-binding protein; PHB, polyhydroxybutyrate; qRT-PCR, quantitative RT-PCR.

promoter region. We also demonstrate that RpoS positively controls transcription of *phbB* and *phbR*.

METHODS

Microbiological procedures. Bacterial strains and plasmids used are listed in Table 1. Medium and growth conditions were as follows. *A. vinelandii* was grown at 30 °C in peptone yeast medium (PY), or Burk's nitrogen-free salts medium (BS) (Kennedy *et al.*, 1986) supplemented with 2 % sucrose. *E. coli* strains DH5 α and BL21 were grown on Luria-Bertani medium (LB) at 37 °C. Antibiotic concentrations used (in $\mu\text{g ml}^{-1}$) for *A. vinelandii* and *E. coli*, respectively, were as follows: tetracycline, 20 and 20; kanamycin (Km), 5 and 30; ampicillin (Ap), not used and 100; nalidixic acid (Nal), 20 and 20; streptomycin (Sm), 2 and 20; and gentamicin (Gm), 1.5 and 10. *A. vinelandii* transformation was carried out as described by Page & von Tigerstrom (1978); Bali *et al.* (1992). PHB production was measured as described previously (Segura & Espín, 1998).

Construction of *A. vinelandii* mutants. Primers phbRupper and phbRlower (Table 1) were used to amplify a PCR fragment containing the complete *phbR* gene using chromosomal DNA of strain UW136 as template. This fragment was digested with *NdeI* and *XhoI* restriction enzymes and cloned into the pTYB1 plasmid (NEB). The resultant plasmid, pMC1, was digested with *NcoI* (a single *NcoI* restriction site is present 375 bp downstream from the ATG start codon). The ends of the *NcoI*-linearized pMC1 were made blunt by treatment with Klenow fragment (Fermentas) and used for cloning an *SmaI* fragment containing the Km-resistance cassette from vector pBSL97. The resultant plasmid, pMC2, was linearized with the *NdeI* restriction enzyme and transformed into the UW136 and JGW-S strains, generating the *phbR* mutant AHW1 and the *rpoS-phbR* double mutant AHW2, respectively. The presence of the *phbR::Km* mutation in these strains was confirmed by PCR (data not shown).

The *scrX* gene encodes a protein with sequence identity to α -glucosidases. Loss of *scrX*, either by replacement or by interruption using an antibiotic-resistance gene, did not affect the capacity of *A. vinelandii* to grow using sucrose as the sole carbon source, indicating that the *scrX* gene product is not essential for sucrose catabolism (Johnson *et al.*, 2006). Therefore, the *scrX* gene was used as a neutral region for integration of the *phbR-gusA* gene fusion. Primers scrX-F and scrX-R (Table 1) were used to amplify a 2956 bp fragment containing the *scrX* gene. This fragment was cloned into pJET1.2 to give plasmid pAHX. Primers phbRint and B2 (Table 1) were used to amplify a 3.2 kb fragment containing the *phbR::gusA*-Gm gene fusion from strain JGW-R (Peralta-Gil *et al.*, 2002). This fragment was cloned within the *EcoRI* site (made blunt with Klenow fragment) present in the *scrX* gene in plasmid pAHX. The resultant plasmid, pMC4, was linearized with *XhoI* and transformed into the UW136, AHW1, JGW-S and AHW2 strains. Transformants of each strain carrying *phbR-gusA*-Gm within the *scrX* gene were isolated and named AHW5, AHW6, AHW7 and AHW8, respectively. The presence of the *phbR-gusA*-Gm fusion within *scrX* was confirmed by PCR analysis (data not shown).

Primers Rfin and phbBa12 (Table 1) were used to amplify a 966 bp fragment containing the *phbB* gene from UW136. This fragment was cloned into plasmid pJET1.2, producing plasmid pAHB. A 2.8 *XhoI* fragment, carrying the *gusA*-Gm cassette, was isolated from plasmid pSM-Gus-Gm, made blunt with the Klenow fragment, and cloned (in the same orientation as the *phbB* gene) into the unique *SmaI* site present within the *phbB* gene of pAHB to produce plasmid pAHB-A, carrying the *phbB::gusA*-Gm fusion. Plasmid pAHB-A was transformed into the UW136, AHW1, JGW-S and AHW2 strains. Transformants of each strain carrying the *phbB::gusA*-Gm fusion

were isolated and named AHW9, AHW10, AHW11 and AHW12, respectively. The presence of the *phbB::gusA* gene fusion was confirmed by PCR (data not shown).

Complementation of the *phbR* and *rpoS* mutants. Plasmids pAHR-KM and pAHS-GM, carrying wild-type copies of the *phbR* and *rpoS* genes, respectively, were constructed as follows. A 1902 bp fragment containing the *phbR* gene was amplified from UW136 using primers bmut2 and compphbRRev, and cloned into plasmid pJET1.2 to produce plasmid pAHR. A Km cassette from plasmid pBSL97 was inserted into the *XbaI* site of pAHR. The resultant plasmid, pAHR-KM, unable to replicate in *A. vinelandii*, was transformed into strain JGW-R. A transformant resistant to Gm and Km, having plasmid pAHR-KM integrated into the chromosome, was isolated and named ASW3. PCR analysis showed that co-integration of pAHR-GM in ASW3 occurred between the *phbR* promoter and the Gm-resistance cassette (see Fig. 2b), thus allowing the wild-type *phbR* gene to be transcribed from its own promoter.

Primers compRpoSFw and compRpoSRev and chromosomal DNA from strain UW136 were used to amplify a 2549 bp fragment carrying the *rpoS* gene, which was cloned into pJET1.2, to produce plasmid pAHS. The Gm cassette from plasmid pBSL98 was cloned into the *XbaI* site of pAHS to produce plasmid pAHS-GM, which was introduced into strain JGW-S by transformation to produce strain AHW4, carrying pAHS-GM integrated between the *rpoS* promoter and the Sp cassette (see Fig. 2c).

Nucleic acid procedures. DNA isolation and cloning, and random primer procedures were carried out as described by Sambrook *et al.* (1989). DNA sequencing was done with a Perkin Elmer/Applied Biosystems DNA sequencer. The sequences of all the primers used in this work are shown in Table 1.

Quantitative RT-PCR (qRT-PCR). Expression of *phbB*, *phbR* and *rpoS* was measured by qRT-PCR, as previously reported (Noguez *et al.*, 2008). For RNA extraction, the cultures were grown in Burk's liquid medium supplemented with 2 % sucrose as the carbon source. Cells were collected at the logarithmic or stationary phase of growth. The primers used for the qRT-PCR assays (Table 1) were as follows: phbBFw/phbBRev for *phbB*; phbRFw/phbRRev for *phbR*; RTuprpoS/RTdownrpoS for *rpoS*; and gyrAFw/gyrARev for *gyrA*, which was used as internal control to normalize the results. All assays were performed in triplicate. The data are presented as fold changes of mRNA levels of the mutant strain relative to those of the wild-type.

Purification of maltose-binding protein (MBP)-PhbR. To express and purify PhbR, a 1047 bp fragment corresponding to the *phbR* gene was amplified by PCR using *Taq* Platinum High Fidelity Polymerase (Invitrogen), and phbRmalFw and phbRmalLw primers. The resultant PCR product was digested with *HindIII* and *EcoRI*, and ligated into plasmid pMAL-C2X (NEB). The ligation mix was transformed into *E. coli* DH5 α , and a transformant carrying plasmid pMC3 was isolated. This plasmid was sequenced to confirm the presence of an intact *phbR* gene and transformed into *E. coli* BL21 (Invitrogen). Expression of the MBP-PhbR in strain BL21/pMC3 was induced by the addition of IPTG (0.3 mM). After 2 h induction, protein purification was performed at 4 °C under non-denaturing conditions using an amylose resin column, as described in the manufacturer's protocol (NEB). The MBP-PhbR protein was maintained in storage buffer (10 mM Tris/HCl, 50 mM potassium chloride, 1 mM DTT, 0.5 mM EDTA, pH 8.0). The protein was concentrated utilizing Microcon YM-10 centrifugal filters (Amicon) and stored at 4 °C. Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951), using BSA as a standard. SDS-PAGE of the purified protein revealed the expected molecular mass of approximately 45 kDa (data not shown).

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Description or sequence (5' to 3')	Source or reference
<i>A. vinelandii</i> strains		
UW136	Wild-type	Laboratory collection
JGW-S	UW136 with an <i>rpoS</i> ::Sp mutation	Peralta-Gil <i>et al.</i> (2002)
JGW-R	UW136 with a <i>phbR</i> ::Gm mutation	Peralta-Gil <i>et al.</i> (2002)
AHW1	UW136 with a <i>phbR</i> ::Km mutation	This study
AHW2	JGW-S with a <i>phbR</i> ::Km mutation	This study
ASW3	JGW-R with pAHR-KM co-integrated	This study
AHW4	AHW2 with pAHR-GM co-integrated	This study
AHW5	UW136 with a <i>phbR</i> :: <i>gusA</i> fusion	This study
AHW6	AHW1 with a <i>phbR</i> :: <i>gusA</i> fusion	This study
AHW7	JGW-S with a <i>phbR</i> :: <i>gusA</i> fusion	This study
AHW8	AHW2 with a <i>phbR</i> :: <i>gusA</i> fusion	This study
AHW9	UW136 with a <i>phbB</i> :: <i>gusA</i> fusion	This study
AHW10	AHW1 with a <i>phbB</i> :: <i>gusA</i> fusion	This study
AHW11	JGW-S with a <i>phbB</i> :: <i>gusA</i> fusion	This study
AHW12	AHW2 with a <i>phbB</i> :: <i>gusA</i> fusion	This study
<i>E. coli</i> strains		
DH5 α	<i>supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
BL21 BL21(DE3)	F ⁻ <i>ompT hsdS_B (r_B m_B) gal dcm</i>	Invitrogen
Plasmids		
pJET1.2	Cloning vector	Fermentas
pMAL-C2	Expression vector	NEB
pTYB1	Cloning vector	NEB
pBSL97	Plasmid with a Km cassette	Alexeyev <i>et al.</i> (1995)
pBSL98	Plasmid with a Gm cassette	Alexeyev <i>et al.</i> (1995)
pSM-Gus-Gm	Plasmid with a <i>gusA</i> ::Gm cassette	Peralta-Gil <i>et al.</i> (2002)
pTC2P	Plasmid with <i>phbR-phbB</i> intergenic region	Peralta-Gil <i>et al.</i> (2002)
pMC1	pYTB1 derivative with <i>phbR</i>	This study
pMC2	pMC1 derivative with <i>phbR</i> ::Km mutation	This study
pMC3	pMAL-C2X derivative with <i>phbR-malE</i> fusion	This study
pMC4	pAHX derivative with <i>phbR</i> :: <i>gusA</i> ::Gm	This study
pAHX	pJET1.2 derivative with <i>scrX</i>	This study
pAHB	pJET1.2 derivative with <i>phbB</i>	This study
pAHB-A	pAHB derivative with <i>phbB</i> :: <i>gusA</i> ::Gm	This study
pAHR	pJET1.2 derivative with the <i>phbR</i> locus	This study
pAHS	pJET1.2 derivative with the <i>rpoS</i> locus	This study
pAHR-KM	pAHR derivative with a Km cassette	This study
pAHS-GM	pAHS derivative with a Gm cassette	This study
Oligonucleotides		
gyrAFw	CCAGCAAGGGCAAGGTCTA	Noguez <i>et al.</i> (2008)
gyrARev	TCGTCCAGCGGCAACAGGT	Noguez <i>et al.</i> (2008)
phbRFw	CCACATGCGTTTGGTTTCTTG	Noguez <i>et al.</i> (2008)
phbRRev	TCTGCCCTAAGCGTCTCCTC	Noguez <i>et al.</i> (2008)
phbBFw	TCCGCAATCCAAACGCAAAG	Noguez <i>et al.</i> (2008)
phbBRev	GTGCTCTCCCAATCGGTTAC	Noguez <i>et al.</i> (2008)
RTuprpoS	AGGATGTCCTGGACGATGAG	Cocotl-Yañez <i>et al.</i> (2011)
RTdownrpoS	TCCAGCGCCCTAGTGTAGTC	Cocotl-Yañez <i>et al.</i> (2011)
emsaPsup	CGAGCCGTCAGTTTGTGC	Cocotl-Yañez <i>et al.</i> (2011)
psrAprim	CGAGGTCTCGGCCGAATCCC	Cocotl-Yañez <i>et al.</i> (2011)
phbRmalFw	TAGCGAATTTCATGTTTACAGTCAGT	This study
phbRmalLw	GATCAAGCTTTTCATCCGTATGCGAC	This study
scrX-F	GTGGTGCAACGACTCTGC	This study
scrX-R	TCGCGGACCGTGCCATATG	This study
Rini	CAAATCCCGCCTCGCCAAGC	This study
Rmed	CATCGCTCTCGGCATTCTCC	This study
Rfin	CCGATAGCAAAAAGCCACTG	This study

Table 1. cont.

Strain, plasmid or oligonucleotide	Description or sequence (5' to 3')	Source or reference
B1	GGCTCATTCTCGATTGGGTC	This study
B2	GTGGCTTTTGTCTATCGGGC	This study
B3	GGATGGAGAATGCCGAGAGC	This study
bmut2	CTTCCTTTTGTGCGGAG	This study
comp ϕ h ϕ RRev	TCTCCTGCATAGTGACGG	This study
comp ρ SFw	CAATTTCTCAGTATGGGC	This study
comp ρ SRv	TAGGCATGCAGGGAGTGG	This study
phbRint	CTTGAGCCCTATATCCGGATT	This study
phbRupper	GGCTCATATGTTTACAGTCAGT	This study
phbRlower	CCAACTCGAGTCCGTATGCGAC	This study
phbBal2	CTAGCAGCTTTCCGAATG	This study
foot ϕ h ϕ B4	TTTTTTGTGCGGAGACCCTGG	This study

DNA gel mobility shift assays. DNA fragments corresponding to the intergenic *phbR*–*phbB* region were amplified from *A. vinelandii* UW136 chromosomal DNA by PCR using primers Rini, Rmed, Rfin, B1, B2 and B3. DNA-binding reactions were carried out in a total volume of 20 μ l. The reactions contained DNA-binding buffer [10 mM Tris/HCl, 50 mM KCl, 1 mM DTT, 0.5 mM EDTA, 5 % (v/v) glycerol, 10 μ g BSA μ l⁻¹], 0.1 nmol of each DNA fragment labelled with (γ -³²P) δ ATP, and variable amounts of PhbR (0–1 μ M). As a negative control, a 263 bp fragment of DNA corresponding to the promoter region of *psrA* was used (Cocotl-Yañez *et al.*, 2011). This fragment was obtained by PCR using primers emsaPsup and psrAprm. DNA-binding reactions were carried out at room temperature for 25 min, and the samples were subjected to native PAGE in 6 % polyacrylamide gels in a buffer containing 90 mM Tris, 90 mM H₃BO₃ and 2 mM EDTA. The gel was dried, and radioactive signals were detected by autoradiography.

DNase I footprinting assays. Plasmid pTC2P was used as a template for PCR amplification of the *phbB* 5' regulatory region. A ³²P-labelled oligonucleotide complementary to the 5' coding region of *phbB* (foot ϕ h ϕ R4), and an oligonucleotide complementary to the –109 to +78 bp region relative to the transcriptional start ρ _{B1} (Rfin), were used. The binding of MBP–PhbR to the labelled DNA fragment was performed at room temperature in buffer containing 10 mM Tris/HCl, 50 mM KCl, 0.5 mM EDTA, 1 mM DTT, 5 % (v/v) glycerol and 10 mg BSA⁻¹. The reaction mixture was treated with 0.02 U DNase I (Fermentas) for 2 min. The DNase footprinting with MBP–PhbR was performed at 30 °C. The DNA segments were separated by electrophoresis in 7 % polyacrylamide/8 M urea gels alongside sequencing ladders.

RESULTS

Transcription of *phbB* and *phbR* in exponential and stationary phase cultures

In *A. vinelandii*, the levels of the RpoS protein and of the PHB polymer significantly increase during the stationary phase (Sandercock & Page, 2008; Martínez *et al.*, 1997). We determined the level of *rpoS* mRNA by qRT-PCR in exponential and stationary cells of strain UW136 grown on PY medium. As shown in Fig. 1, the level of the *rpoS* transcript was about 35-fold higher in stationary phase cells than in exponentially growing cells. Thus, it was expected

that in the UW136 strain, the levels of the *phbB* and/or *phbR* mRNAs would be higher in stationary phase cells than in exponentially growing cells. Indeed, the levels of *phbR* and *phbB* were four- and eightfold higher, respectively, in stationary phase cells, as determined by qRT-PCR (Fig. 1). These data are in agreement with a role for RpoS as a positive regulator of *phbB* and *phbR* transcription.

PHB accumulation and *phbB* transcription in the *phbR* and *rpoS* mutants

Inactivation of either *phbR* or *rpoS* in strain UW136 reduced accumulation of PHB (Table 2, Fig. 2a). We carried out complementation of the *phbR* and *rpoS* mutant strains.

Fig. 1. Relative mRNA expression for *phbR*, *phbB* and *rpoS* in *A. vinelandii* strain UW136. qRT-PCR analysis of the expression of *phbR* (black bars), *phbB* (white bars) and *rpoS* (grey bars) in exponential (8 h) and stationary phase cells (48 h). The value of the mRNA level of each gene was normalized according to the level of *gyrA* mRNA. The data represent the means of triplicates; error bars, SD. The data are presented as the fold change of mRNA levels of stationary phase cells relative to exponentially growing cells.

Fig. 2. Complementation of JGW-R and JGW-S mutant strains by co-integration of plasmids pAHR-KM and pAHS-GM carrying the *phbR* and *rpoS* genes, respectively. (a) Opacity phenotype (PHB accumulation) of UW136, JGW-R and JGW-S, and the corresponding complemented strains ASW3 and AHW4. (b, c) Schematic representations of the integration of plasmid pAHR-KM into the chromosome of strain JGW-R (b) and pAHS-GM into the chromosome of strain JGW-S (c).

Table 2. PHB production

PHB was determined in cells grown on PY medium for 72 h.

Strain	Genotype	PHB content [mg (mg protein) ⁻¹]
UW136	Wild-type	1.550 ± 0.220
JGW-R	<i>phbR</i> :: <i>gusA</i> -Gm	0.144 ± 0.070
JGW-S	<i>rpoS</i> :: <i>Sp</i>	0.167 ± 0.010
AHW2	<i>rpoS</i> :: <i>Sp</i> / <i>phbR</i> :: <i>gusA</i> -Gm	0.004 ± 0.002
ASW3	<i>phbR</i> :: <i>gusA</i> -Gm/ <i>phbR</i> +	2.490 ± 0.174
AHW4	<i>rpoS</i> :: <i>Sp</i> / <i>rpoS</i> +	2.510 ± 0.273

Plasmids pAHR-KM and pAHS-GM, carrying wild-type copies of the *phbR* and *rpoS* genes and unable to replicate in *A. vinelandii*, were transformed into the JGW-R and JGW-S strains, respectively, for integration into the chromosome. Transformants ASW3 and AHW4 were isolated, and they showed the wild-type PHB accumulation phenotype (Fig. 2, Table 2), confirming that the PHB-defective phenotype was due to the *phbR* and *rpoS* mutations.

To further study the roles of RpoS and PhbR in PHB synthesis, we constructed strain AHW2, an *rpoS*-*phbR* double mutant. As expected, PHB synthesis was abrogated in the AHW2 strain (Table 2), indicating that together, RpoS and PhbR are essential for PHB synthesis. The level of *phbB* mRNA in strains *phbR*, *rpoS* and the double mutant *rpoS*-*phbR* was also determined by qRT-PCR. Fig. 3(a) shows that in the absence of either RpoS or PhbR, the levels of the *phbB* transcripts were significantly reduced in stationary cultures. No *phbB* mRNA was detected in the *rpoS*-*phbR* double mutant.

The effect of the *rpoS* and *phbR* mutations on *phbB* expression was also analysed using a *phbB*-*gusA* gene fusion. The induction kinetics of *phbB* transcription were determined *in vivo* by measuring the β -glucuronidase activity in strain AHW9, a UW136 derivative carrying the *phbB*-*gusA* fusion, and their derivatives AHW10, AHW11 and AHW12, carrying *phbR*, *rpoS* and *rpoS*-*phbR* mutations respectively. The β -glucuronidase activity was determined during growth on PY sucrose medium, as shown in Fig. 3(b). Growth curves were very similar in all strains carrying the *phbB*-*gusA* gene fusion. As shown in Fig. 3(c), β -glucuronidase activity in strain AHW9 increased upon entrance into the stationary phase, in agreement with a role for RpoS as a regulator of *phbB* transcription. Fig. 3(c) also shows that the *rpoS*, *phbR* and *rpoS*-*phbR* mutations significantly reduced the β -glucuronidase activity, in agreement with the reduction in the levels of *phbB* mRNA observed in the *phbR*, *rpoS* and *rpoS*-*phbR* mutant strains.

Transcription of *phbR*

The level of *phbR* mRNA was determined by qRT-PCR in the *rpoS* mutant. As shown in Fig. 4(a), the *rpoS* mutation reduced the concentration of the *phbR* transcript by 95%,

Fig. 3. PhbR and RpoS as positive regulators of *phbB*. (a) qRT-PCR analysis of *phbB* gene expression in *phbR*, *rpoS* and *rpoS-phbR* mutants. The data are presented as fold changes of mRNA levels of *rpoS* (white bar), *phbR* (grey bar) and *rpoS-phbR* mutant strains relative to that of the wild-type UW136 (black bar). (b) Growth of strain AHW9 (▲), and the isogenic *phbR* (■), *rpoS* (●) and *rpoS-phbR* (◆) strains. (c) β -Glucuronidase activity of a *phbB-gusA* gene fusion in strain AHW9 (▲), and in the isogenic *phbR* (■), *rpoS* (●) and *rpoS-phbR* (◆) strains. A magnification of β -glucuronidase activity of mutant strains is shown in the inset.

indicating the requirement for RpoS in the transcription of *phbR*.

The presence of sequences R5–R6, proposed to be PhbR-binding sites and located upstream of *phbR* (Fig. 5a), suggested that PhbR may also act as an autoregulator. We therefore determined the effect of *phbR* mutation on the levels of *phbR* mRNA, as determined by qRT-PCR. As shown in Fig. 4(a), the *phbR* transcript was reduced by about 80 % in the absence of PhbR, suggesting that it also acts as an autoactivator. In the *rpoS-phbR* double mutant, no *phbR* transcript was detected, indicating that both regulatory proteins, PhbR and RpoS, are required for *phbR* transcription.

The effect of RpoS and PhbR on expression of *phbR* was further evaluated using a *phbR::gusA* gene fusion. Strain AHW5, a UW136 derivative carrying the wild-type *phbR* gene and a *phbR::gusA* gene fusion integrated into the chromosome within the *scrX* locus, and strain AHW6, a derivative of AHW5 in which the wild-type *phbR* copy is interrupted with a Km-resistance cassette, were constructed as described in Methods. The β -glucuronidase activity was determined during growth on PY sucrose medium Fig. 4(b). As shown in Fig. 4(c), the β -glucuronidase activity was higher in the strain carrying the wild-type *phbR* copy, while in strain AHW6, the absence of *phbR* reduced the β -glucuronidase activity. This result further supports the contention that PhbR acts as a positive autoregulator.

The positive effect of RpoS on *phbR* expression was also confirmed by the reduction of the β -glucuronidase activity in the AHW7 strain, which carries the *rpoS* mutation (Fig. 4c). Additionally, the *rpoS-phbR* mutant strain (AHW8) showed an activity similar to that of the AHW7 strain, supporting the importance of RpoS and PhbR in *phbR* expression.

Binding of PhbR to the *phbB* promoter regions

Sequence analysis of the *phbR-phbB* intergenic region revealed two almost identical 18 bp sequences, named R1 and R2, centred at positions –134 and –155 upstream of the ATG start codon, where R1 overlaps the –35 region of p_{B1} . These sequences have previously been proposed to be PhbR-binding sites (Peralta-Gil *et al.*, 2002). Other less conserved 18 bp sequences, designated R3, R4, R5 and R6, are present in this region. The R5 and R6 sequences overlap the p_{R2} promoter (Peralta-Gil *et al.*, 2002; Fig. 5a). To determine whether PhbR binds to the *phbB* and *phbR* promoter regions, the PhbR protein was purified as described in Methods. A 405 bp DNA fragment (F1) corresponding to the intergenic *phbB-phbR* region, as well as fragments of 108 (F2), 147 (F3) and 133 bp (F4) containing the R1–R2, R3–R4 and R5–R6 sites, respectively, were amplified by PCR and subjected to an electrophoretic mobility shift assay (EMSA) with purified PhbR. Fig. 5(b, c) shows the binding of PhbR to the 405 nt fragment spanning the *phbR-phbB* intergenic region, as

Fig. 4. PhbR and RpoS as positive regulators of *phbR*. (a) qRT-PCR analysis of *phbR* gene expression in the *phbR* and *rpoS* mutants. The data are presented as fold changes of mRNA levels of *rpoS* (white bar), *phbR* (grey bar) and *rpoS-phbR* mutant strains relative to that of strain AHW5 (black bar). (b) Growth of wild-type UW136 (▲), and of the isogenic *phbR* (■), *rpoS* (●) and *rpoS-phbR* (◆) strains. (c) β -Glucuronidase activity of a *phbB-gusA* gene fusion in strain AHW5 (▲), and in the isogenic *phbR* (■), *rpoS* (●) and *rpoS-phbR* (◆) strains.

well as to the R1–R2-containing fragment F2. Binding of PhbR to the fragments containing the R1–R2 sites was observed at a PhbR concentration of 1.0 μ M (Fig. 5b, c). Some binding of PhbR to the larger fragment F1 was observed at a lower PhbR concentration of 0.2 μ M (Fig. 5b). No binding of PhbR to fragments containing R3–R4 and R5–R6 sites was detected (data not shown), nor to non-specific *psrA* DNA (RR*psrA*) used as a negative control. These results indicate that PhbR activates expression of *phbB* by binding specifically to the promoter region containing the R1 and R2 sequences.

In vitro footprinting analysis

DNase footprinting was performed to determine specific binding of PhbR to the direct repeated sequences TGTCACCAA-N₄-CACTA and TGTCACCAA-N₄-CAGTA (R1 and R2 sites) present in the –35 to –70 bp region upstream of the *p_B1* transcriptional start site for *phbB* (Fig. 6a). As shown in Fig. 6(b), PhbR protected the R1–R2 box at 0.5 μ M, corroborating the data generated by EMSA and indicating that PhbR binds specifically within the R1–R2 boxes. Sequences located upstream and downstream of the PhbR-binding boxes R1–R2 were not protected, even at higher PhbR concentrations (1.0–4.0 μ M).

DISCUSSION

In general, negative regulators bind to promoters, interfering directly with RNA polymerase. In contrast, positive regulators bind to the upstream regions of the promoter, helping to recruit the polymerase and to start transcription (Browning & Busby, 2004).

PhbR belongs to the AraC family of transcriptional activators, some of which bind 18–20 nt repeated sequences. We show here that PhbR binds specifically to the 18 bp R1 and R2 sites, where R1 overlaps the –35 region of the *p_B1* promoter (Fig. 6a). The position of the *A. vinelandii* R1 and R2 sites is similar to the position of some of the binding sites for MelR in the *melAB* promoter of *E. coli*. MelR binds four target sites, named 1', 1, 2' and 2, where site 2' also overlaps the –35 region (Belyaeva *et al.*, 2000). MelR binding to the less conserved site (2') is weak and occurs only in the presence of melibiose (Belyaeva *et al.*, 2000). Modification of the base sequence of this site removes the requirement for melibiose for MelR binding (Tamai *et al.*, 2000). In *A. vinelandii*, the R1 site is almost identical to the R2 site, suggesting that no inducer is required for activation of *phbB* by PhbR.

The data presented in this study, together with previously published data (Peralta-Gil *et al.*, 2002), confirm that PhbR activates the *phbB* gene.

In *E. coli*, the *melR* gene is expressed from a divergent promoter upstream of the *melAB* promoter. MelR also binds to a site that overlaps the –10 region of the *melR*

Fig. 5. PhbR protein binds the *phbB* promoter region. (a) Schematic representation of the *phbR-phbB* intergenic region, showing the position of the *phbB* p_{B1} and p_{B2} and *phbR* p_{R1} and p_{R2} promoters (transcription start sites) and the proposed PhbR-binding sites R1 to R6 (Peralta-Gil *et al.*, 2002). The PCR F fragments used for the EMSA for the binding of PhbR are depicted. (b, c) EMSA showing the binding of various concentrations of PhbR (0.05–1 μ M) to the F1 fragment (b) or to the F2 fragment (c). Stars show PhbR–DNA complexes. (d) Competition assay for PhbR–F1 complex formation to verify the binding specificity. Competition reactions used specific unlabelled competitor (F1 fragment) at the concentrations shown. (e) Competition assay for PhbR–F2 complex formation to verify the binding specificity. Competition reactions used specific unlabelled competitor (F2 fragment) at the concentrations shown.

Fig. 6. (a) The *phbB* promoter region. The R1 and R2 sequences are boxed. The -10 regions for p_{B1} and p_{B2} are underlined. (b) DNase I footprinting analysis of the interaction of PhbR with the *phbB* promoter region. The reaction mixture was treated as described in Methods, and the sequencing ladder was generated with plasmid pCT2P and oligonucleotide footphbR4. –, Absence of PhbR. The -10 regions of the p_{B1} and p_{B2} promoters are indicated. The arrows show the start sites for *phbB* transcription. The R1 and R2 sequences protected from degradation are also shown.

promoter to downregulate its own expression (Wade *et al.*, 2000; Samarasinghe *et al.*, 2008).

The results presented here indicate that PhbR autoregulates its own expression positively. This regulation may be carried out in a direct manner, by binding to the R5 and R6 sites present upstream of the p_{R1} promoter region. However, no specific binding of PhbR to a fragment carrying R5 and R6 sites was observed *in vitro* in the EMSA assays. This result indicates either that PhbR indirectly activates its own transcription, or that a metabolite is required for PhbR binding to these less conserved R5 and R6 sites, similar to the requirement for melibiose in the binding of MelR to the less conserved site 2 (Belyaeva *et al.*, 2000).

PhbR orthologues are present in *Azotobacter* strain FA8 (Pettinari *et al.*, 2001) and in *Pseudomonas* sp. strain 61-3 (Matsusaki *et al.*, 1998). In these bacteria, direct repeat sequences of 18 bp similar to the R1 and R2 PhbR-binding sites are present upstream of the PHB biosynthetic genes, which are also organized in a *phbBAC* cluster. Thus, in these bacteria, activation of the PHB biosynthetic operon by PhbR is expected to occur as in *A. vinelandii*.

Similar to PhbR, RpoS plays an important role in the activation of transcription of the *phbB* and *phbR* genes, as indicated by the negative effect of the *rpoS* mutation on the levels of *phbB* and *phbR* mRNAs and the kinetics of transcription of *phbB::gusA* and *phbR::gusA* gene fusions, showing that induction of transcription occurs upon entrance into the stationary phase in an RpoS-dependent manner.

These observations are in agreement with previously published data that show a significant reduction of the *phbR* transcript levels initiated from the p_{R2} promoter in the *rpoS* mutant (Peralta-Gil *et al.*, 2002). Whether RpoS directly activates *phbR* remains to be investigated. No RpoS-recognized sequences are present in the -10 sequences of the p_{R1} or p_{R2} promoters (Peralta-Gil *et al.*, 2002), suggesting that RpoS control of *phbR* expression is indirect.

The control of PHB synthesis by RpoS is also in agreement with previous observations that indicate that in *A. vinelandii*, accumulation of *rpoS* mRNA, as well as PHB, occurs mainly in cells entering the stationary phase and during the stationary phase (Castañeda *et al.*, 2001; Sandercock & Page, 2008; Martínez *et al.*, 1997).

We propose that the activation of *phbR* expression by RpoS allows an increase in the concentration of PhbR in the stationary phase, which in turn further increases its concentration by autoactivation (in the presence of an inducer), allowing activation of the *phbBAC* biosynthetic operon and the synthesis of PHB in the stationary phase.

ACKNOWLEDGEMENTS

A.H.-E. thanks CONACYT for financial support during his PhD studies. M. C. thanks DGAPA for a postdoctoral fellowship. We thank Daniel Segura for critical reading of the manuscript.

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Edited by: M. F. Hynes