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Oxygen-Sensitive Global Regulator, Anr, Is Involved in the Biosynthesis of Poly(3-Hydroxybutyrate) in *Pseudomonas extremaustralis*

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Key Words
Anr · Poly(3-hydroxybutyrate) · Microaerobiosis · *Pseudomonas*

Abstract
We analyzed the influence of the redox global regulator Anr on the accumulation of poly(3-hydroxybutyrate) (PHB) in *Pseudomonas extremaustralis*. Anr regulates a set of genes in the aerobic-anaerobic transition including genes involved in nitrate reduction and arginine fermentation. An *anr* mutant was constructed using PCR-based strategies. The wild-type strain was able to grow in both microaerobic and anaerobic conditions using nitrate as the terminal electron acceptor while the mutant strain was unable to grow under anaerobic conditions. In bioreactor cultures, PHB content in the wild-type strain was higher in microaerobic and anaerobic cultures compared with highly aerated cultures. The mutant strain showed decreased PHB levels in both aerobic and microaerobic conditions compared with the wild-type strain. Inactivation of *anr* led to decreased expression of *phaC* and *phaR* genes as demonstrated in real-time RT-PCR experiments. Associated with the PHB gene region, two putative binding sites for Anr were found that, in line with the phenotype observed in bioreactor cultures, suggest a role of this regulator in PHB biosynthesis.

Introduction

Polyhydroxyalkanoates (PHAs) are reduced carbon storage compounds that are accumulated by many bacterial species during unbalanced growth conditions. Accumulation and degradation of PHAs endow bacteria with enhanced survival, competition abilities, and stress tolerance, thereby increasing fitness in changing environments [Ayub et al., 2009; Kadouri et al., 2005; López et al., 1995; Pham et al., 2004; Ruiz et al., 2004]. PHAs exhibit thermoplastic and elastomeric features in addition to other interesting properties, including biodegradability and biocompatibility that make them promising industrial materials [Hazer and Steinbüchel, 2007]. Poly(3-hydroxybutyrate) (PHB) is the most common and most studied of these polymers.

In order to improve polymer production, several strategies have been developed based on both the use of natural PHA bacterial producers or different *Escherichia coli* recombinant strains. In our laboratory, a recombinant *E. coli* strain carrying the *pha* genes from *Azotobacter* sp. FA8 with additional mutations in the redox regulator ArcA was studied. In this strain PHB production was higher in microaerobic conditions due to an increase in the NADH pool of the cells [Nikel et al., 2006].

The switch from aerobic to anaerobic metabolism is accompanied by repression of a number of genes linked

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Table 1. Similarity of the product of *anr* gene of *P. extremaustralis* to other bacterial proteins

Gene	Organism	Amino acid similarity to other gene products, %		
		amino acid number	identity	similarity
anr (AF053611.1)	*P. fluorescens* Pf-5	244	97	98
anr (YP_347543)	*P. fluorescens* PfO-1	244	95	97
anr (AAN69845)	*P. putida* KT2440	244	92	96
anr (AAA25713.1)	*P. aeruginosa* PAO1	244	87	94
cydR (YP_002799173.1)	*Azotobacter vinelandii*	244	86	98
fnrA (CAA81129)	*P. stutzeri* ATCC 14405	244	84	95
fnr (NP_415850.1)	*Escherichia coli* K12	250	47	72

to aerobic respiration while genes responsible for anaerobic respiration or fermentation are induced [Mazoch and Kucera, 2001]. In *E. coli* these adaptative responses are mainly coordinated by ArcA and Fnr [Gunsalus, 1997]. Fnr and members of the Fnr family bind as homodimers to palindromic sequences of DNA and contain a $[4Fe\text{-}4S]^{+2}$ cluster that supports an active dimeric form. Oxygen is considered a direct Fnr effector, the cluster suffers an oxidation reaction resulting in a $[2Fe\text{-}4S]^{+2}$ inactive monomeric form [Crack et al., 2003]. In *Pseudomonas*, a transcriptional factor, Anr, shares structural and functional similarity to Fnr [Sawers, 1991]. Anr is essential for nitrate respiration and arginine fermentation [Galimand et al., 1991] and hydrogen cyanide and heme synthesis in anaerobiosis [Blumer and Haas, 2000; Rompf et al., 1998]. It is involved in biofilm development [Yoon et al., 2002], aerotaxis [Hong et al., 2004], survival under anaerobic stress conditions [Boes et al., 2008] and in the expression of several transcriptional regulators like Dnr [Rompf et al., 1998]. Recently, the role of Anr in the expression of multiple terminal oxidases in *Pseudomonas putida* in aerobiosis was described [Ugidos et al., 2008].

Pseudomonas extremaustralis is a highly stress-resistant novel bacterial species isolated from an Antarctic environment [López et al., 2009] that is able to synthesize PHB from octanoate but not from glucose [Ayub et al., 2006]. Genetic analysis demonstrated that *pha* genes are organized in a cluster, containing genes *phaR*, *phaB*, *phaA* and *phaC*, and that impaired PHB production from glucose is due to a defective β-ketothiolase *(phaA)* gene [Ayub et al., 2006]. It was also found that PHA biosynthesis genes in this species are located within a genomic island [Ayub et al., 2007].

Based on the knowledge that PHA accumulation is affected by global regulators involved in oxygen limitation response in recombinant *arcA E. coli* mutants [Nikel et al., 2006], we suggest that Anr could affect PHB accumulation in *P. extremaustralis*. In this work, we investigated the influence of oxygen availability on *P. extremaustralis* growth and the role of the Anr global regulator on PHB accumulation.

Results

Cloning and Characterization of the Role of anr on Microaerobic Metabolism in P. extremaustralis

The *anr* gene of *P. extremaustralis* was cloned and sequenced using PCR strategies. A 1,100-bp amplicon was obtained containing the whole *anr* sequence (738 bp) and a fragment of adenine phosphoribosyltransferase gene *(apt)* that is also found downstream of *anr* in this and in other *Pseudomonas* species. The deduced amino acid sequence of *P. extremaustralis* Anr showed a conserved size (245 aa) and high identity with the *anr* gene products of several *Pseudomonas* species and with the homologous protein CydR of *Azotobacter vinelandii*. It also showed 47% identity with the anaerobic transcriptional regulator Fnr of *E. coli* (table 1). Additionally, in silico analysis of the deduced amino acid sequence revealed that it had three conserved cysteins (Cys) residues in the amino-terminal region and one internal Cys involved in the binding to the $[4Fe\text{-}4S]^{+2}$ cluster, as well as a DNA-binding domain located in a helix-turn-helix (HTH) structure in the C-terminal region. This HTH motif contains Glu-209 and Ser-212 conserved residues (*E. coli* numbering), which are required for the binding of Fnr to DNA. These

motifs are similar to Fnr homologues found in other *Pseudomonas* [Laville et al., 1998].

Functionality of the *anr* gene of *P. extremaustralis* was tested by complementation of an *E. coli* MG1655 Δ*fnr* strain using plasmid pGANR. In *E.coli,* Fnr activates the formate lyase, which catalyzes the CO_2 and H_2 formation during glucose fermentation and it also controls nitrate reductase [Soupene et al., 2003]. McConkey lactose nitrate agar (MCLNA) in anaerobiosis is used to characterize Fnr-negative phenotype by observing size and color of colonies. Gas formation was observed in *E. coli* complemented with the *anr* of *P. extremaustralis* in glucose media after 2 days of culture without agitation, and not in the Fnr-negative strain. Complementation with pGANR also restored the wild-type Fnr phenotype showing big, pink colonies in MCLNA under anaerobiosis in comparison with small, dark red colonies of the mutant strain. Anr of *P. extremaustralis* was able to complement the *E. coli* Fnr mutant, similarly to that reported for other *Pseudomonas* Fnr-like regulators [Galimand et al., 1991].

Some experiments were performed in order to test Anr-regulated phenotypes in *P. extremaustralis* under low oxygen tension. Several *Pseudomonas* species, including *P. aeruginosa,* are described as denitrifiers [Yoon et al., 2002]. *P. extremaustralis* was able to grow under microaerobic conditions using nitrate as an alternative electron acceptor. However, *P. extremaustralis* produced high amounts of nitrite (248 ± 3 mg/l) after 24 h of culture in hermetic bottles and no gas was detected in the Durham tube after 7 days. The control strain *P. aeruginosa* PAO1 produced gas in unshaken nitrate-containing medium under the same conditions. Results suggested that *P. extremaustralis* was able to perform nitrate reduction but not denitrification. Some *Pseudomonas* species are able to use arginine as a terminal electron acceptor in a fermentation reaction. Arginine allowed slow growth of *P. extremaustralis* when a terminal electron acceptor was lacking, as reflected by slight increase in the number of colony-forming units after 72 h incubation in completely filled bottles compared with time zero ($1.7 \cdot 10^7$ vs. $2.3 \cdot 10^7$ cfu/ml). In absence of this amino acid, bacterial counts decreased by one order of magnitude (up to $1.7 \cdot 10^6$ cfu/ml) at the end of the experiment.

Characterization of the P. extremaustralis anr *Mutant*

A *P. extremaustralis anr*$^-$ mutant was constructed using PCR deletion methods. This strain, which contains a 250-bp deletion and a Km cassette insertion in *anr*, was unable to grow in anaerobic conditions and to use nitrate as an electron acceptor. Nitrite was not detected in the supernatant of cultures after 24 h incubation. In accordance, the mutant strain failed to use arginine to sustain survival (data not shown). The mutant also showed impaired growth in aerobiosis in 0.5 NE2 supplemented with octanoate (NEO). This low nitrogen medium is used to improve PHB accumulation in *Pseudomonas* strains [Huisman et al., 1992]. Addition of 0.3% (w/v) casein amino acids to this culture medium (NEOC) restored growth of the mutant strain. This phenomenon was also reported in the *E. coli arcA* mutant [Fu et al., 1991].

Growth on Bioreactor Cultures

To study the influence of Anr on PHB accumulation, bioreactor cultures were carried out in *P. extremaustralis* and its *anr* mutant using different aeration conditions in NEO supplemented with nitrate (NEON). Growth of the wild-type strain in casein amino acid-supplemented cultures (NEONC) was similar in aerobiosis and microaerobiosis ($\mu = 0.72$ and $\mu = 0.80\ h^{-1}$) reaching an $OD_{600\ nm} = 7$, while poor growth was observed under strict anaerobic conditions (fig. 1). The wild type was also able to grow in NEON reaching slightly lower values in aerobiosis, microaerobiosis and anaerobiosis, (5.5, 5 and 0.4 $OD_{600\ nm}$, respectively) in comparison with NEONC.

The mutant strain showed very poor growth under aerobic conditions in NEON and it was unable to grow on this culture medium on microaerobic or anaerobic conditions. In contrast, the mutant was able to grow under aerobic conditions in NEONC, but with a reduced growth rate ($\mu = 0.32\ h^{-1}$) under these highly aerated conditions compared with the wild-type strain (fig. 1). Under microaerobiosis, the growth of this strain was strongly affected as evidenced by a sharp decrease in OD (fig. 1). In strict anaerobiosis, as expected, the mutant strain was unable to grow (fig. 1).

Nitrite accumulation in casein amino acid-supplemented media was detected in microaerobic and anaerobic cultures of the wild-type strain reaching values of 11 ± 2 mg/l and 137 ± 20 mg (mean ± SD), respectively, after 24 h culture. These values were higher compared to those obtained in aerobic conditions where nitrite concentration was only 1.7 ± 0.1 mg/l. In contrast, the *anr* mutant showed much lower nitrite accumulation than the wild-type strain, with values ranging between 0.1 and 0.2 ± 0.01 mg/l for aerobic and microaerobic conditions, as expected in a nitrate-reducing negative phenotype.

PHB Accumulation in Bioreactor Cultures

Although growth of the wild-type strain in NEONC was similar in both aerobic and microaerobic conditions,

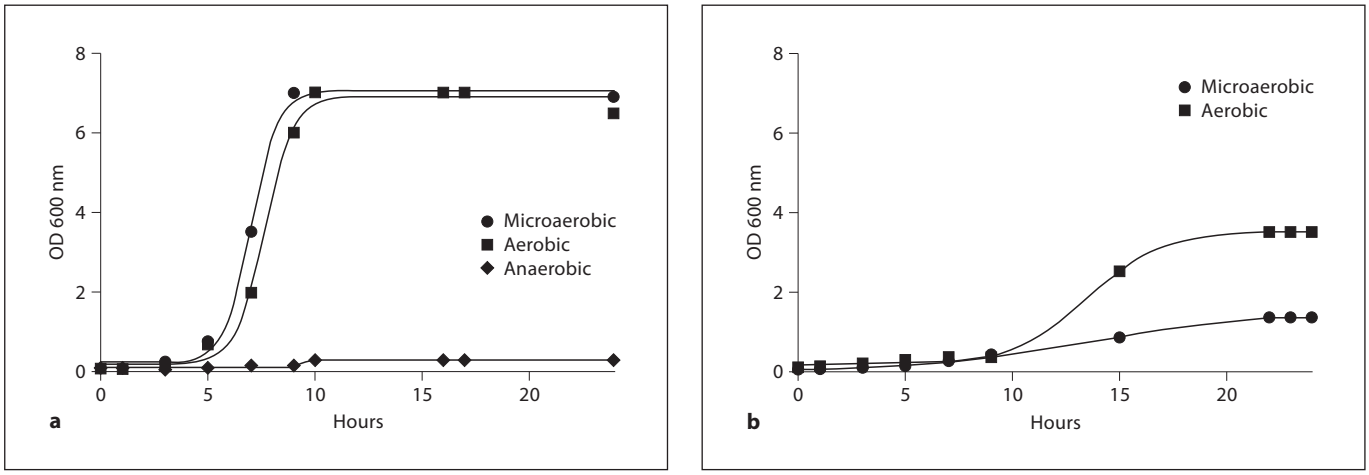

Fig. 1. Growth of *P. extremaustralis* and its *anr* mutant under different oxygen conditions in bioreactor cultures. **a** *P. extremaustralis*. **b** *P. extremaustralis anr*⁻. Cells were grown in NEONC at 28°C under different aeration conditions. Aerobic and microaerobic cultures were performed at 95–100% and 15–20% of air saturation, respectively. Anaerobic cultures were performed with a saturated atmosphere of N_2. All curves are representative of two independent experiments.

Table 2. PHB production of wild-type and *anr* mutant of *P. extremaustralis* in bioreactor cultures with and without casein amino acids (CAS) addition

Condition	*P. extremaustralis*		*P. extremaustralis anr*⁻	
	without CAS	plus CAS	without CAS	plus CAS
Aerobic	2.57 ± 0.2	7.38 ± 0.42	0.00 ± 0.3	1.00 ± 0.2
Microaerobic	37.84 ± 0.4	51.33 ± 0.24	NG	21.66 ± 0.82
Anaerobic	18.42 ± 1.8	16.69 ± 0.23	NG	NG

PHB content is expressed as percentage of cellular dry weight. Cultures were performed in 0.5 NE2 medium supplemented with octanoate and nitrate (NEON). NG = No growth.

PHB content, measured after 24 h of bioreactor culture, was considerably higher in microaerobiosis than in aerobiosis (table 2), reaching a value of 51.33% cell dry weight. The wild-type strain was also capable of producing PHB under strict anaerobic conditions (table 2). Without casein amino acids, the PHB content showed the same pattern as in NEONC with values slightly lower in the three aeration conditions studied (table 2). In the *anr* mutant, the polymer was poorly detected in aerobiosis while in microaerobiosis it was markedly lower compared with the wild-type strain (table 2). These results suggested a role of Anr in the regulation of the amount of PHB accumulated.

Analysis of the Upstream Region of the PHB Biosynthetic Genes

In a previous work, we reported two putative PhaR-binding sites, designated as R1 and R2, associated with the intergenic *phaR–phaB* region and a –10 and –35 promoter sequence located downstream of them in *P. extremaustralis* [Ayub et al., 2006]. Further analysis of this region showed two conserved Fnr-like boxes. An Anr-box named Anr-box1 (TTGAC....ATCAA) was found overlapping the –10 sequence. The second Anr-box named Anr-box2 was found upstream [Ayub, 2009, PhD thesis], in position –115 relative to the start codon of the *phaB* gene (CTGAT....ATCAA) (fig. 2a). Both putative

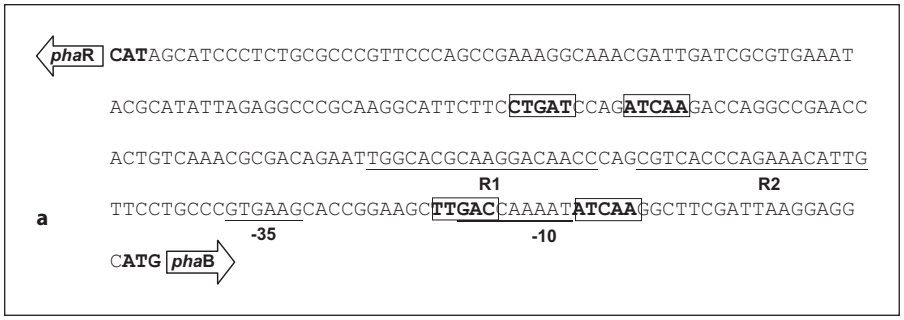

Fig. 2. a Organization of the *P. extremaustralis* intergenic *phaR–phaB* region showing putative Anr boxes. The sequences –35 and –10 of a probable σ^{70} promoter and putative PhaR-binding sites (R2 and R1) are also indicated on the nucleotide sequence. The *phaB* and *phaR* start codons are shown by boldface type and arrows indicate the direction of the transcription. Anr boxes are boxed. **b** Alignment of putative Anr-boxes found in *phaR–phaB* intergenic region of *P. extremaustralis* with other Fnr-like boxes. The name of the gene located downstream from the binding site is indicated in brackets.

b

TTGAC.....ATCAA	*ANR-box 1 (phaB)P. extremaustralis*
CTGAT.... ATCAA	ANR-box2 (*phaB*) *P. extremaustralis*
GTGAC.... ATCGA	CydR-box (*phaB*) *Azotobacter vinelandii*
TTGAC.... ATCAG	*ANR-box (arc*D) *P. aeruginosa*
TTGAT.... ATCAA	FNR-box (consensus) *Escherichia coli*

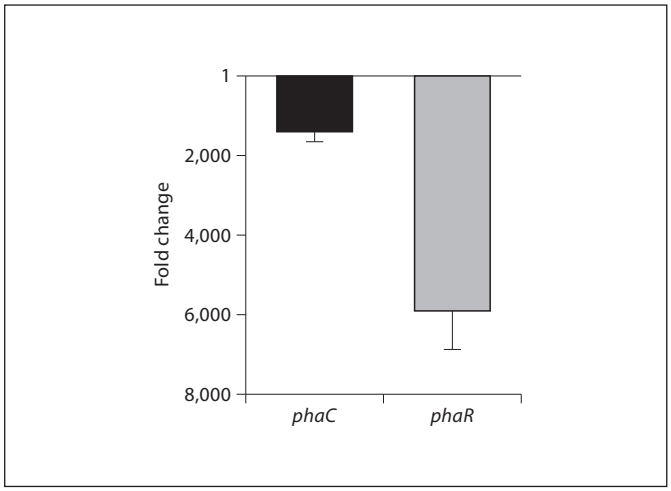

Fig. 3. Effect of *anr* mutation on expression of *phaR* and *phaC* genes. Values (expressed as fold change) indicate the change in the mRNA levels of genes encoding the PHB proteins upon inactivation of the *anr* gene in comparison with wild type strain, which correspond a value of 1. Cells were grown under microaerobic conditions. mRNA levels were determined by real-time RT-PCR as indicated in the Experimental Procedures section. Values represent the average of three independent RNA preparations, analyzing each sample in triplicate. Error bars indicate the standard deviation of the three independent assays.

Anr boxes of *P. extremaustralis* were similar to the binding site for the homologous protein CydR found in *Azotobacter vinelandii* that is also located in the intergenic *phaR–phaB* region (fig. 2b). The Anr boxes were also similar to the consensus Fnr-box of *E. coli* and to the sequence of the Anr box located in the *arcD* promoter of *P. aeruginosa*, one of most studied anaerobic inducible genes in this species (fig. 2b).

Effect of Anr on Expression of phaR *and* phaC *Genes*

To test the hypothesis that inactivation of *anr* could negatively affect PHB biosynthesis, real-time RT-PCR experiments were carried out with total RNA of microaerobic cultures. The expression of *phaR* and *phaC* genes encoding a putative transcriptional regulator and the PHA polymerase was analyzed. In the *anr* mutant strain, the expression of both genes was significantly lower compared with the wild-type strain (fig. 3), *phaR* and *phaC* showed 6,000- and 1,380-fold decreased expression, respectively. These results strongly suggested that Anr could be involved in PHB biosynthesis probably adding a global regulation to the specific regulation of PHB genes.

Discussion

The *anr* gene of *P. extremaustralis* has been identified and the microaerobic growth of this species has been studied. Results showed that under low oxygen tension

this strain was able to reduce nitrate but not to denitrify. Denitrification was extensively described in *P. aeruginosa*, but some *Pseudomonas* species like *P. putida* KT2440 do not carry out the denitrification process [dos Santos et al., 2004]. Furthermore, *P. fluorescens* CHA0 is incapable of growing under anaerobic conditions in spite of having a functional Anr [Laville at al., 1998]. Growth of the *anr* mutant of *P. extremaustralis* was significantly affected in microaerobic conditions showing the importance of this regulator under low oxygen availability. However, the mutation also affected aerobic growth as the mutant was not able to grow without casein amino acids addition. The influence of Anr on aerobic control of several cytochrome genes was reported in *P. aeruginosa* and *P. putida* KT2440 [Anjana and Willams, 1997; Ugidos et al., 2008]. The poor growth of the *anr* mutant of *P. extremaustralis* could respond to an action of Anr over different genes even under aerobic conditions, as it is a master regulator.

Based on previous studies that showed changes in PHB accumulation in recombinants *E. coli* redox mutants, we analyzed the effect of the *anr* gene on PHB accumulation in *P. extremaustralis*. We found a positive role for Anr, an oxygen-sensitive global regulator, on PHB accumulation in a *Pseudomonas* species. PHB production is a rare or uncommon characteristic among members of this genus [Kessler and Palleroni, 2000] that usually accumulate PHAs composed of medium chain length monomers. *P. extremaustralis* has a set of PHB biosynthetic genes located within a genomic island [Ayub et al., 2006, 2007]. The organization of this *pha* gene cluster was similar to that observed in *Azotobacter* species: *A. vinelandii* and *Azotobacter* sp. FA8 [Peralta-Gil et al., 2002; Pettinari et al., 2001] and also in *Pseudomonas* sp. 61-3 [Matsusaki et al., 1998]. The analysis of intergenic *phaR–phaB* region of *P. extremaustralis* showed two putative Anr boxes, similar to one previously described for the homologous CydR regulator of *A. vinelandii* [Peralta-Gil et al., 2002]. Anr boxes were not detected in the PHB cluster of *Pseudomonas* sp. 61-3. However, we found a putative Anr box upstream of *phaB* in the complete published genome of *P. stutzeri* A1501 [Yan et al., 2008], in which the organization of the PHB genes and the flanking regions are similar to *P. extremaustralis*.

Several Fnr-like boxes show distinct sequences in the same strain [Boes et al., 2008; Winterler and Haas, 1996]. Two boxes were found to be essential for the expression of the aerotaxis gene in *P. aeruginosa* and for the hydrogenase genes in *E. coli* [Hong et al., 2004; Kovacs et al., 2005]. In addition, the functionality of hemi-boxes located inside the *hem*A gene promoter sequence (at –20 position between –10 and –35 sequence) and a box overlapping the –35 promotor sequence of the *ccoN1* cytochrome gene were reported for Anr [Krieger et al., 2002; Ugidos et al., 2008]. If the two slightly different Anr boxes of *P. extremaustralis* are necessary to regulation remain to be determined. However, the presence of the boxes in line with the low PHB observed in the *anr* mutant suggests that this regulator could be involved in a positive control of *pha* genes.

It has previously been reported that some global regulators could affect the expression of PHA genes. RpoS inactivation induce the activity of the promoter of *phaC1* in *P. putida* KT2440 [Raiger and Ruiz, 2008] and AniA in *Sinorhizobium meliloti* is involved in a complex PHB synthesis regulation [Povolo and Casella, 2000]. In *A. vinelandii,* the role of the oxygen-dependent global regulator CydR was studied in relation to PHB production. In the exponential phase of growth, the PHB production increases in a CydR mutant [Wu et al., 2001]. Based on this, recent analysis of the *A. vinelandii* genome suggested a wider role of CydR in the integration of carbon source and oxygen availability in comparison with Anr [Setubal et al., 2009]. Our results suggest that besides its traditional role, Anr could affect the expression of other metabolic genes in *Pseudomonas* species, since the *anr* mutant of *P. extremaustralis* accumulated less PHB, and the wild-type strain showed 90% decreased PHB content under aerobic conditions compared with microaerobiosis, where Anr is in a mainly active condition. Quantitative RT-PCR experiments support the hypothesis that the *anr* mutation affects the expression of PHB genes since a dramatic decrease in *phaR* and *phaC* expression was observed under microaerobic conditions.

Understanding the influence of redox regulators on PHA synthesis in natural producers helps to elucidate the role of PHB on adaptation to extreme conditions derived from oxygen deprivation, and it is also relevant for biotechnological purposes. For example, polymer production could be improved by choosing the appropriate aeration conditions during the fermentation process increasing sustainability. PHA biosynthesis is affected by several factors, including carbon-nitrogen content and reducing equivalents availability, and it can also be controlled by both specific and global regulators.

In this study, we showed for the first time a positive role of an oxygen sensitive protein on PHB synthesis in a *Pseudomonas* species. The presence of two Anr boxes associated with the promoter region of the PHB biosynthetic cluster suggests a possible role of Anr in the regula-

tion of these genes. However, since Anr is a global regulator that affects many metabolic genes, an overlapping physiological effect of this regulator cannot be ruled out.

Experimental Procedures

Bacterial Strains

P. extremaustralis DSM 17835 [López et al., 2009], an Antarctic PHB-producing strain with high stress resistance, was used throughout this study. *P. aeruginosa* PAO1 was used as a positive control for denitrification assays. For *anr* functionality studies, *E. coli* MG1655 and Δ*fnr* derivative strain [Soupene et al., 2003] were used.

Microaerobic Experiments

Experiments were performed in 100-ml hermetically sealed bottles containing 50 or 100 ml of 0.5 NE2 culture medium [Huisman et al.,1992] supplemented with 15 mM sodium octanoate (NEO) as carbon source and either 30 mM arginine or 0.06% (w/v) KNO_3. Bottles were incubated at 28°C and low shaking (75 rpm) to avoid cellular aggregation. The oxygen initially present in the bottle was consumed during growth. Arginine growth experiments were performed in completely filled bottles incubated for 72 h. Bacterial number was measured by colony counts on nutrient agar plates. Denitrification capability was evaluated by observing gas trapped in a Durham tube after 7 days of growth under microaerobic conditions.

Cloning and Molecular Analysis of anr *Gene*

The complete sequence of *anr* was obtained by PCR amplification using the following primers (Anr2 up 5′-CGATTCAAATGTCCGAGCCA-3′ and AnrApt low 5′-AAGCCGCTGATCCTGTTCCGCA-3′) designed by aligning several *anr* sequences belonging to *Pseudomonas* species obtained from databases. The fragment was cloned in pGEM-T-easy (Promega Corporation) resulting in pGANR. The fragment was sequenced (Macrogen Inc., Korea) and the resulting sequence was analyzed using BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST/) and deposited in the EMBL Nucleotide Sequence Database under accession number AM901400.

E. coli Δfnr *Complementation*

Plasmid pGANR was introduced by transformation into competent cells of *E. coli* MG1655 Δ*fnr*. Gas trapped in a Durham tube was tested after 2 days of cultivation in Luria Broth glucose (2% w/v) under microaerobic conditions. *E. coli* MG1655 was used as a positive control. Color and size of colonies grew in McConkey medium supplemented with 0.5% (w/v) nitrate and 1% (w/v) lactose were observed after 48 h of culture in anaerobiosis using an Anaerobic Jar and Anaerocult A (Merck).

Mutant and Recombinant Strains Construction

An *anr* mutant strain was obtained using two steps PCR crossover deletion method described by Link et al. [1997]. PCR mixtures were carried out according to Nikel et al. [2006]. Denaturation at 94°C for 5 min was performed before the amplification was run. The thermal cycle profile was 45 s at 94°C, 30 s at 54°C, and 1 min at 72°C (for a total of 34 cycles) and a final 10 min 72°C extension step. For the first PCR reaction the amino-terminal sequence, primers Anr2up and anrRlow (5′-TTGAT TGGATCCTTTAAGACATCGCTCAGGCTGAATGT CTT-3′) were used. For the carboxy-terminal sequence, the degenerate primer anrLow (5′-CTGCTGGVAAC GGGTGAA-3′) and anrRup (5′-TCTTAAAGGATCCAATCAACGAAGAAGACCG CCGACGAGCG-3′) were used. In the second step to obtain the final 850 bp *anr* product with a 250 bp deletion, the left and right fragments were annealed at their overlapping region and amplified by PCR as a single fragment, using the external primers. A *Bam*HI site was generated in the middle of the fragment. The amplification fragment was cloned in pGEM-T Easy. The plasmid was cut with *Bam*HI and ligated with a kanamycin (Km) cassette obtained from the plasmid pUC4 K (Pharmacia, San Francisco, Calif., USA) cut with *Bam*HI. The resulting plasmid pGDANR, with the incomplete *anr* and the insertion of Km cassette, does not replicate in *Pseudomonas*, was introduced by transformation into competent cells of *P. extremaustralis* [Lee et al., 2005]. Transformants were selected by plating on LB agar containing 50 μg/ml of Km. Km^R colonies were then screened for plasmid loss in ampicillin (Amp) in NEO and 200 μg/ml Amp. Km^R-Amp^S colonies were grown without agitation in the same medium supplemented also with KNO_3 (NEON). Clones impaired to grow in microaerobiosis and unable to produce nitrite were selected. Double recombination event was checked by PCR followed by sequencing.

Bioreactor Cultures

Batch cultures were carried out in a 3.7-liter stirred tank reactor equipped with six flat-bladed disk turbines and proportional-integral-differential (PID) controller (Bioengineering, Switzerland). Starting volume was 1.5 liters of NEON. In some experiments (0.3% w/v) casein amino acids (NEONC) were added. Experiments were carried out for 24 h at 28°C, at pH 7. To prevent foam formation, 30 μl/l Antifoam 289 (Sigma-Aldrich) was manually added at the beginning of the run. Dissolved O_2 concentration (dO_2) was measured using an Ag/AgCl polarometric O_2 probe (Mettler, Switzerland). Aerobic cultures were performed at 95–100% of air saturation with an air flux of 0.2 l/min and 1.8 l/min at the beginning and the end of the experiment, respectively. The stirring was automatically adjusted for air saturation percentage maintenance (between 100 and 700 rpm) by a PID controller installed in the control unit of the bioreactor. Microaerobic cultures were carried out at 15–20% of air saturation with an air flux of 0.06 l/min and 0.9 l/min at the beginning and the end of the experiment, respectively. Stir was also automatically adjusted for air saturation percentage maintenance (between 100 and 700 rpm). In aerobic cultures, air was sparged by using a microsparger that produced very small air bubbles while in microaerobic cultures a sparger with a unique pore which produced large air bubbles was used. Anaerobic cultures were performed with a saturated atmosphere of N_2. Growth was monitored by measuring $OD_{600\ nm}$ at different times. Pellets and supernatant cultures were used for PHB and nitrite measurement, respectively.

Analytical Determinations

PHB content was determined from lyophilized cells subjected to methanolysis and hot chloroform extraction. Methyl ester derivatives were chromatographically analyzed as previously de-

scribed [Braunegg et al., 1978]. The PHB content was expressed as a percentage of cell dry weight. Nitrite production was determined in the supernatant cultures according to Gerhardt et al. [1981].

Real-Time RT-PCR Experiments

Total RNA of *P. extremaustralis* and *P. extremaustralis anr* of microaerobic 24-hour cultures was extracted by using the RNeasy Mini kit (Qiagen) and treated with DNaseI. cDNA was obtained using random hexamers (Promega) and AMV retrotranscriptase following the manufacturer's instructions. At least three independent cultures were analyzed for each strain. Real-time RT-PCR was performed by use a LightCycler (DNA Engine M.J. Research) and SYBR green. Three genes were analyzed using the following primers: *phaC,* 5′-CTTCGTCCTCGGATCTTCTG-3′ and 5′-ATCGACCCACCAACTCCTG-3′; and *phaR,* 5′-ACGAACACCCGAATAACTGC-3′ and 5′-AGAAATCGAGCGTTTGAGGA-3′ and 16S rRNA gene 5′-AGCTTGCTCCTTGATTCAGC-3′ and 5′-AAGGGCCATGATGACTTGAC-3′, employed as reference for normalization of expression levels of PHB genes in each strain. The cycling parameters used were as follows: denaturation at 95°C for 4 min; 40 cycles at 95°C for 25 s, 60°C for 15 s, and 72°C for 15 s; and fluorescence acquisition at 80°C in single mode. Relative change in the expression of individual genes in both strains was analyzed following the standard curve method [Larionov et al., 2005].

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