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Anr, the anaerobic global regulator, modulates the redox state and oxidative stress resistance in *Pseudomonas extremaustralis*

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The role of Anr in oxidative stress resistance was investigated in *Pseudomonas extremaustralis*, a polyhydroxybutyrate-producing Antarctic bacterium. The absence of Anr caused increased sensitivity to hydrogen peroxide under low oxygen tension. This phenomenon was associated with a decrease in the redox ratio, higher oxygen consumption and higher reactive oxygen species production. Physiological responses of the mutant to the oxidized state included an increase in NADP(H) content, catalase activity and exopolysaccharide production. The wild-type strain showed a sharp decrease in the reduced thiol pool when exposed to hydrogen peroxide, not observed in the mutant strain. *In silico* analysis of the genome sequence of *P. extremaustralis* revealed putative Anr binding sites upstream from genes related to oxidative stress. Genes encoding several chaperones and cold shock proteins, a glutathione synthase, a sulfate transporter and a thiol peroxidase were identified as potential targets for Anr regulation. Our results suggest a novel role for Anr in oxidative stress resistance and in redox balance maintenance under conditions of restricted oxygen supply.

INTRODUCTION

Bacterial physiological responses to different growth conditions, such as nutrients and oxygen availability, changes in temperature, and the presence of stress agents are frequently coordinated by a number of different regulatory mechanisms, including global regulatory proteins (Martínez-Antonio & Collado-Vides, 2003). The expression of specific genes, belonging to a wide variety of regulons, responds to the overall physiological economy.

One of the most critical environmental signals for bacteria is oxygen availability, which triggers several specific responses, and affects the cellular redox balance. The redox state of cells in the transition from aerobic to anaerobic conditions, and the regulatory proteins involved have been extensively studied in *Escherichia coli* (Partridge *et al.*, 2007; Tolla & Savageau, 2010). In the opportunistic pathogen *Pseudomonas aeruginosa*, changes in oxygen tension cause an increment in oxidative stress, giving rise to high expression of virulence factors, exoenzymes and quorum-sensing components (Kim *et al.*, 2005; Sabra *et al.*, 2002).

Oxidative stress protection is crucial in aerobic bacteria. Well-developed defence mechanisms against reactive oxygen species (ROS) are present in *P. aeruginosa*, and include two superoxide dismutases (SODs) with either iron or manganese as cofactor, three catalases (KatA, KatB and KatC) and four alkyl hydroperoxide reductases (AhpA, AhpB, AhpCF and Ohr) (Salunkhe *et al.*, 2005). The regulation of these proteins is important during the exposure to oxidative agents produced in respiratory processes and in the presence of antibiotics, macrophages, cold and contaminant compounds (Albesa *et al.*, 2004; Cochran *et al.*, 2000; Lapaglia & Hartzell, 1997).

Sensing of oxygen availability is coordinated by Anr in most *Pseudomonas* species. This transcription factor detects differences in oxygen levels inside the cells by means of an oxygen-sensitive cysteine redox cluster (Galimand *et al.*, 1991). Several genes are under Anr control, either directly or indirectly. These include *nar* (nitrate reductase), *cupA* (fimbriae), *arcDABC* (the arginine deiminase pathway), *hmn* (haem synthesis), *aer* (proteins involved in aerotaxia), and the *cioA*, *ccoN* and *ccoO* cytochromes (Ray & Williams, 1997; Trunk *et al.*, 2010). Several phenotypic and genetic

Abbreviations: CDW, cell dry weight; DCFH$_2$-DA, dichlorodihydrofluorescein diacetate; PHA, polyhydroxyalkanoate; ROS, reactive oxygen species; SOD, superoxide dismutase.

The GenBank/EMBL/DDBJ accession numbers for the complete genome sequence of *P. extremaustralis* are AHIP01000001–AHIP01000135.

characteristics related to microaerobic physiology have been studied in *P. aeruginosa*, which is able to perform complete denitrification (O'Callaghan *et al.*, 2011, 2012; Schreiber *et al.*, 2007; Sonnleitner *et al.*, 2011).

Pseudomonas extremaustralis was recently isolated from an Antarctic environment (López *et al.*, 2009). This species shows high stress resistance and is able to synthesize large amounts of poly(3-hydroxybutyrate), the best-known member of a bacterial reserve polymers family known as polyhydroxyalkanoates (PHAs) (Ayub *et al.*, 2004, 2009). PHAs are involved in bacterial survival under different stressful conditions (López *et al.*, 1995; Kadouri *et al.*, 2005). *P. extremaustralis*, a non-pathogenic bacterium, is an interesting model as it not only shares general characteristics with representative *Pseudomonas* species, but also presents metabolic differences from *P. aeruginosa*, such as nitrite accumulation during microaerobic growth (Tribelli *et al.*, 2010).

There have been many studies concerning the relationship between oxygen and metabolism, but there is no information on the influence of Anr on the redox ratio for *Pseudomonas* species and very few reports have analysed antioxidative defences under low oxygen availability. Due to the importance of oxidative stress and cellular redox balance in bacterial physiology, we analysed the effect of Anr on these responses, revealing that this regulator has a crucial role in the modulation of components involved in redox homeostasis and antioxidative defences.

METHODS

Bacterial strains and growth conditions. *P. extremaustralis* DSM 25547 and an *anr* mutant containing a 250 bp deletion and a kanamycin cassette insertion in this gene were used throughout this study (Tribelli *et al.*, 2010, 2012). A complemented strain was constructed by inserting the entire wild-type *anr* sequence and a 900 bp DNA fragment upstream of the coding region into the chromosome by using a mini-Tn*5* delivery system (de Lorenzo *et al.*, 1990). The upstream sequence contains a putative promoter for *anr* and a 700 bp sequence corresponding to the terminal region of the *hemN* gene. This fragment was cloned in the *Not*I site of plasmid pUT-mini-Tn*5*. This system allowed the transposition of the antibiotic cassette and the *anr* gene. The plasmid was introduced in the *anr* mutant strain by conjugation and the transconjugants were selected in Luria–Bertani (LB) agar plates supplemented with 15 μg tetracycline (Tet) ml^{-1} and 50 μg kanamycin (Km) ml^{-1}. Positive clones showing similar aerobic and microaerobic growth in comparison with the wild-type strain were tested for nitrite accumulation in LB medium supplemented with Tet and Km and 0.08 % (w/v) KNO_3 and incubated under microaerobic conditions for 48 h.

Batch cultures were carried out in a 3.7-litre stirred tank bioreactor equipped with a proportional-integral-differential controller (Bioengineering). The working volume was 1.5 l $0.5NE_2$ medium (Huisman *et al.*, 1991) supplemented with 0.25 % (w/v) sodium octanoate, 0.08 % (w/v) KNO_3 and 0.3 % (w/v) casein amino acids (Difco). Cultures were carried out for 24 h at 28 °C. The dissolved O_2 concentration was measured using an Ag/AgCl polarometric O_2 probe (Mettler). Aerobic and microaerobic culturing was performed as described previously (Tribelli *et al.*, 2010). Briefly, aerobic bioreactor cultures were made at 95–100 % air saturation with an air flux of 0.2 and 1.8 l min^{-1} at the beginning and the end of the experiment, respectively. Microaerobic bioreactor cultures were made at 15–20 % air saturation with an initial air flux of 0.06 l min^{-1} and a final flux of 0.9 l min^{-1}. The stirring speed was automatically adjusted to between 100 and 700 r.p.m. to maintain the percentage of air saturation in both conditions.

Aerobic cultures were developed in Erlenmeyer flasks with shaking at 250 r.p.m. and using a 1 : 10 medium-to-flask volume ratio. Microaerobic cultures were developed in hermetically sealed bottles using a 1 : 2 medium-to-flask volume ratio with gentle shaking (75 r.p.m.). For each experiment, an overnight culture developed in 500 ml Erlenmeyer flasks containing 100 ml culture medium was used to prepare inocula.

Growth was monitored by following OD_{600} at selected times throughout the culture. Samples were taken at 24 h and centrifuged at 4 °C at 3000 ***g***. Bacterial pellets were used for analytical determinations. The biomass concentration was expressed as cell dry weight (CDW) per litre of culture.

Stress experiments. Aerobic and microaerobic 24 h cultures were performed for each strain and sensitivity to H_2O_2 was evaluated as described previously (Ayub *et al.*, 2004) using sterile Whatman no. 1 filter discs (6 mm) impregnated with 5 μl of 30 % (v/v) H_2O_2 (Merck). Zones of inhibition were measured after incubation at 30 °C for 24 h. In addition, the growth rate in the presence of sublethal concentrations of H_2O_2 was determined in cultures performed in 96-well microtitre plates (Nunc) at 30 °C with gentle shaking (100 r.p.m.). Twofold serial dilutions were performed starting from a maximal H_2O_2 concentration of 1700 μM. OD_{600} was monitored for 24 h, and control experiments without H_2O_2 were also included. The percentage of growth inhibition was calculated as $100-[(\mu\ H_2O_2/\mu\ \mathrm{control})\times 100]$, taking into account the specific growth rate (μ) to avoid differences between the strains. Three independent experiments were carried out, with duplicate measurements in each one.

Cellular redox state measurement. The $NADH/NAD^+$ and $NADPH/NADP^+$ ratios were quantified in the pellet fraction of triplicate 1 ml bioreactor culture samples. Samples were transferred to pre-cooled plastic tubes and the metabolic activity was quenched by immersion of the tubes in liquid N_2. Samples were stored for no longer than 24 h at −70 °C until enzyme analysis. Aliquots of 1 ml of thawed samples were treated with 300 μl of either 0.2 M HCl [NAD(P)H extraction] or 0.2 M NaOH [$NAD(P)^+$ extraction]. Acid/alkaline extraction was carried out at 50 °C for 10 min, and samples were rapidly placed on ice to cool them at 0 °C afterwards. Suspensions were neutralized by dropwise addition of 1 M HCl or NaOH, and cellular debris was removed by centrifuging at 14 000 ***g*** for 5 min. Supernatants were then transferred to new tubes and immediately used for co-factor measurements. Nucleotide determination was performed as described by Bernofsky & Swan (1973), using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as the final electron acceptor, but as modified by Gibon & Larher (1997) and Nikel *et al.* (2008). The dinucleotide content was normalized to the CDW.

Oxygen consumption. Oxygen consumption was measured with a Clark-type polarographic electrode (Biological O_2 Monitor, model 53; Yellow Springs Instruments Inc.). The assay mixture contained 0.2 % (w/v) glucose in Ringer solution [0.65 % (w/v) NaCl), 0.042 % (w/v) KCl, 0.025 % (w/v) $CaCl_2$], and it was equilibrated for 3 min at 30 °C under magnetic stirring in an open measurement chamber. Aerobic shaken-flask or microaerobic bottle cultures were used to prepare a cell suspension (OD_{600} of *ca.* 0.5). This suspension was injected into the chamber, which was immediately closed. The total volume in the chamber was 4.5 ml and O_2 uptake was expressed as the variation in the percentage of O_2 saturation min^{-1} (mg protein)$^{-1}$. Protein

concentration was determined by the Folin phenol reagent method (Lowry *et al.*, 1951) using BSA as standard.

Flow cytometry analysis. ROS were measured in 24 h aerobic shaken flask and microaerobic bottle cultures. The OD_{600} was adjusted to 0.2 and incubated under the same conditions for 1 h with 3 mM H_2O_2. This concentration was similar to those used in previous studies (Salunkhe *et al.*, 2005; Wei *et al.*, 2012). Cultures were centrifuged, cell number was adjusted to approximately 1×10^7 cells ml^{-1}, 50 μM dichlorodihydrofluorescein diacetate ($DCFH_2$-DA; Invitrogen) was added and the cultures were incubated for 1 h at 30 °C. The cells were washed once and resuspended in 400 μl PBS. A similar procedure was carried out to detect reduced thiol molecules in microaerobic cultures, by adding 1 μM Mercury Orange dye (Sigma). A FACS AriaI BD cell sorter (BD, Bioscience) equipped with an FIT-C filter (Blue Diode at 488 nm) and an FL2 (585–542 nm) filter was used. At least 10 000 events were measured in each tube. The autofluorescence of the bacterial population was detected with non-stained cells and used to determine the positive fluorescence. The geometric mean (Gm) as well as the percentage of cells with positive fluorescence signal (%positive cells) were determined using WindMDI 2.9 software (Joseph Trotter).

Real-time RT-PCR experiments (RT-qPCR). Total RNA of *P. extremaustralis* and the *anr* strain from 24 h bioreactor cultures was extracted by using the RNeasy Mini kit (Qiagen) and treated with DNase I. cDNA was obtained using random hexamers (Promega) and AMV retrotranscriptase following the manufacturer's instructions. At least three independent cultures were analysed for each strain. RT-qPCR was performed by using a LightCycler (DNA Engine M.J. Research) and Real-Time PCR mix (EvaGreen qPCR Mix Plus, no Rox). Gene expression of *cioA*, corresponding to the cyanide-insensitive oxidase, and *ccoN*, which belongs to the terminal oxidase Cbb3-1 of *Pseudomonas putida* KT2440 (termed Cbb3-2 in *Pseudomonas aeruginosa*), was analysed using the following primers: 5′-TGGTTCACCACCGAAATCGGGC-3′ and 5′-GTCGATTCGCTGCCTTCGTTGAT-3′, and 5′-GATCGTGGGCGCCGGCATTA-3′ and 5′-ACCACGGCGTAGACCACCCA-3′, respectively. To normalize the expression levels in each strain, the 16S rRNA gene was also amplified using primers 5′-AGCTTGCTCCTTGATTCAGC-3′ and 5′-AAGGGCCATGATGACTTGAC-3′. The cycling parameters used were as follows: denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 25 s and 61.5 °C for 15 s; and fluorescence acquisition at 80 °C in single mode. Relative changes in the expression of individual genes in both strains were obtained through the relative standard curve method (Larionov *et al.*, 2005).

Catalase activity measurement. Catalase activity was measured as described by Aebi (1984). Briefly, cells from microaerobic bottle cultures were disrupted by sonication followed by centrifugation at 20 000 ***g*** at 20 °C. The supernatant was used to monitor the decomposition of H_2O_2 at 240 nm. An enzymic unit is defined as the amount of enzyme capable of decomposing 1 μmol H_2O_2 min^{-1} (mg protein)$^{-1}$.

Exopolysaccharide content. Exopolysaccharides were measured using anthrone reagent (Quelas *et al.*, 2006). Briefly, 1 ml of supernatant obtained from bioreactor cultures was centrifuged for 40 min at 21 500 ***g*** at 4 °C. The supernatant was transferred to a clean tube and three volumes of absolute ethanol were added. The mixture was incubated overnight at −20 °C and centrifuged for 40 min at 21 500 ***g*** at 4 °C. The pellet was resuspended in 100 μl of 0.5 M NaCl. Aliquots of this preparation were used to determine polysaccharides using a reagent consisting of 0.2 % (w/v) anthrone in 95 % (w/v) H_2SO_4. The absorbance of the reaction mixture was measured at 620 nm (Trevelyan & Harrison, 1956) and glucose was used as standard. The results were expressed as g glucose (100 g CDW)$^{-1}$.

Bioinformatic analysis. The Anr regulon of *P. extremaustralis* was determined using the Virtual Footprint tool available in the PRODORIC software (http://prodoric.tu-bs.de). Putative target genes were considered only when the Anr-box was located within 400 bp upstream from the start ATG codon, based on previous data with experimental support (Trunk *et al.*, 2010).

Nucleotide sequence accession numbers. The complete genome sequence of *P. extremaustralis* has been deposited at GenBank/EMBL/DDBJ under accession nos AHIP01000001–AHIP01000135 (Tribelli *et al.*, 2012).

Statistical analysis. The significance of each treatment was evaluated by Student's *t* test with confidence levels at >95 % (i.e. $P<0.05$ was considered as significant). For flow cytometry experiments, the median value and the 25th and 75th percentiles are shown. In these experiments, the significance was tested with the Mann–Whitney *U* test at $P<0.05$.

RESULTS

Resistance to oxidative stress under microaerobic conditions is reduced in the *anr* mutant

Oxidative stress resistance was evaluated by exposing cells from aerobic and microaerobic cultures to H_2O_2. While cells from aerobic cultures of both strains showed a similar resistance to H_2O_2 (with inhibition zones of 1.90 ± 0.13 cm for the wild-type strain and 2.10 ± 0.04 cm for the *anr* strain, $P>0.05$), the *anr* mutant presented a significantly lower resistance than the wild-type strain in microaerobic cultures (with inhibition zones of 2.85 ± 0.06 cm for the wild-type strain mutant and 3.31 ± 0.11 cm for the *anr* strain, $P<0.05$). The stress resistance phenotype was rescued in the mutant strain complemented with *anr*, which showed an inhibition zone similar to the wild-type (2.90 ± 0.11 cm), and significantly different from that obtained for the mutant strain ($P<0.05$). In addition, we evaluated growth of the strains in the presence of different concentrations of H_2O_2 in liquid cultures. Growth inhibition in the presence of H_2O_2 was higher for the *anr* mutant than the wild-type strain at concentrations above 400 μM (Table 1).

Table 1. Effect of the addition of H_2O_2 in microaerobic cultures.

Values represent the mean ± SD of triplicate measurements.

H_2O_2 concentration (μM)	Growth inhibition (%)	
	Wild-type strain	*anr* mutant
212	0 ± 0.2	0 ± 0.3
425	0 ± 0.1	15.1 ± 0.3*
850	57.6 ± 2.3	93.3 ± 1.5*
1700	73.2 ± 0.2	97.1 ± 1.6*

*Significant differences ($P<0.05$) using Student's *t* test.

To further analyse this phenotype, we determined the ROS content in the presence and absence of H_2O_2 by flow cytometry, staining the cells with $DCFH_2$-DA, an ROS-sensitive probe. Under aerobic conditions, the proportion of cells that gave positive fluorescence, either in the presence or in the absence of H_2O_2, was similar for the wild-type and the *anr* mutant strains (Fig. 1a). In addition, Gm values did not show any significant differences between the wild-type [15.8 (14.5, 20.4) and 15.5 (13.3, 16.6) with or without H_2O_2, respectively] and its *anr* derivative in both analysed conditions [17.3 (16.4, 21.0) and 15.1 (13.6, 16.1) with or without H_2O_2, respectively].

In microaerobic cultures, the ROS-positive cell population was higher for the mutant strain than for the wild-type (Fig. 1b), with Gm median values of 14.4 (11.8, 16.5) and 12.7 (10.5, 14.2), respectively. The complemented mutant strain presented a Gm value of 14.2 (13.74, 14.50) and an ROS-positive cell value similar to that found in cultures of the wild-type strain [17.7 (10.5, 26.1)]. In addition, when H_2O_2 was added to the microaerobic cultures, the percentage of ROS-positive cells showed significant differences ($P<0.05$, Fig. 1b), with higher Gm values for the *anr* mutant [118 (66.2, 121.9)] than for the wild-type strain [15.9 (15.1, 16.6)]. In these conditions, the percentage of ROS-positive cells [20.3 % (19.5, 20.9)] and the Gm value [18.9 (18.3, 21.6)] in the complemented *anr* strain were similar to those observed for the wild-type strain, but significantly different from those of the *anr* mutant ($P<0.05$).

Taken together, these results indicate that the ROS level in the mutant was higher than in the parental strain under microaerobic conditions, in agreement with a lower resistance to oxidative stress, which are both reversed in the complemented mutant.

Anr is necessary to maintain redox balance

Respiratory activity is the most prominent source of ROS, and maintaining a balanced redox state is crucial for cellular functions, especially during stress injury.

The intracellular redox state was investigated in the two conditions of oxygen supply by measuring the content of reduced and oxidized nicotinamide adenine dinucleotides. As expected, the $[NADH+NADPH]/[NAD^+ + NADP^+]$ ratio was lower under aerobic than under microaerobic conditions for all strains tested. In aerobiosis, the redox quotient was 0.68 ± 0.11 for the wild-type and 0.41 ± 0.11 for the *anr* strain ($P<0.05$). However, the difference between both strains was higher for cells cultivated under low oxygen tension, with the value of the redox quotient being 0.94 ± 0.08 for the wild-type and 0.51 ± 0.03 for the *anr* strain ($P<0.05$), thus suggesting an impairment in the control of redox balance, mainly under limited oxygen conditions. In addition, *anr* cells growing in microaerobiosis presented a $[NADP^+ + NADPH]$ pool of 8.67 ± 0.11 μmol (g CDW)$^{-1}$, while in the wild-type strain the value for this parameter was 5.59 ± 0.39 μmol (g CDW)$^{-1}$. A similar trend was observed under aerobic growth conditions, as the $[NADP^+ + NADPH]$ content reached 7.72 ± 0.24 μmol (g CDW)$^{-1}$ in the *anr* mutant and 6.63 ± 0.43 μmol (g CDW)$^{-1}$ in the wild-type strain, probably suggesting a metabolic preference for NADP(H) over the non-phosphorylated form of the nucleotide in the *anr* mutant.

To determine if the differences observed in the redox state were related to changes in the respiratory activity, oxygen consumption ability was measured in wild-type and mutant cells from cultures carried out at different aeration conditions. Oxygen consumption [expressed as Δ% oxygen saturation min^{-1} (mg protein)$^{-1}$] in cells from aerobic cultures was similar for both strains, with a slightly higher

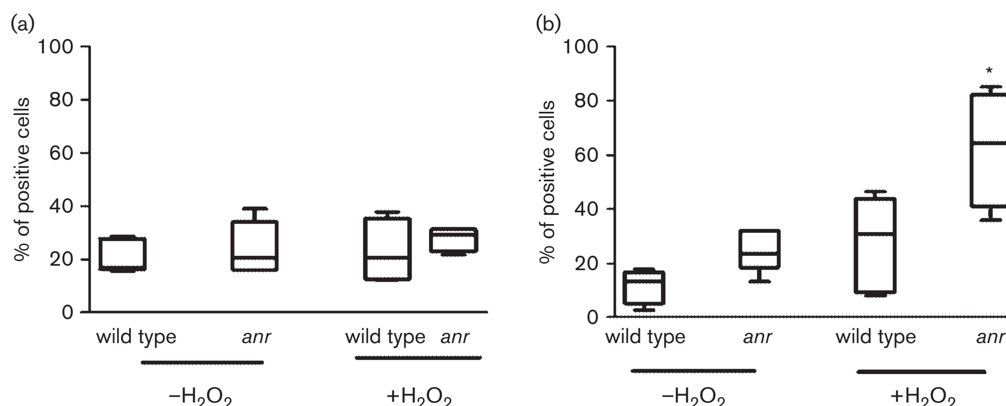

Fig. 1. Analysis of the ROS content in cells from aerobic or microaerobic cultures of *P. extremaustralis* and its *anr* derivative. Quantification of cells positive for ROS signal was performed by using flow cytometry analysis of aerobic cells (a) and microaerobic cells (b). Values represent the median Gm value and the 25th and 75th percentiles of three independent experiments with three replicates for each. The asterisk (*) denotes significant differences ($P<0.05$) using the Mann–Whitney *U* test.

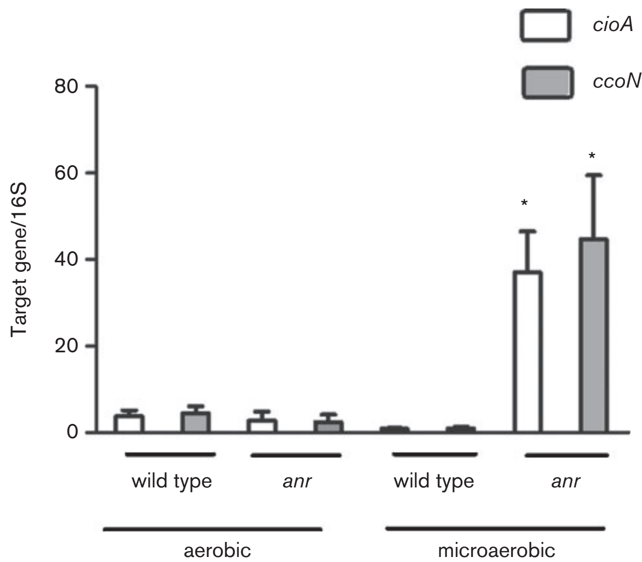

Fig. 2. Effect of *anr* mutation on the expression of *cioA* and *cooN* genes in aerobic and microaerobic bioreactor cultures. Values were expressed as the ratio between the raw level of expression of each target gene and the 16S rRNA gene, and represent the mean ± SD of three independent experiments. The asterisk (*) denotes significant differences ($P<0.05$) using Student's *t* test.

value for the wild-type strain (8.56 ± 1.71) than for the *anr* mutant (5.32 ± 0.54), although the difference was not significant ($P>0.05$). In contrast, under microaerobic growth conditions, *anr* mutant cells showed a higher oxygen consumption ability (14.70 ± 2.07) as compared with the wild-type strain (4.56 ± 1.02, $P<0.05$), suggesting an alteration in the respiratory activity in the absence of Anr.

The expression of cytochrome-coding genes (*cioA* and *ccoN*) was also analysed through RT-qPCR experiments. The *cioA* and *ccoN* genes presented an Anr-box located at positions −170 and −98, respectively, relative to the start codon. We found that under aerobic conditions the expression of *cioA* and *ccoN* was similar in both strains. However, the expression of both cytochrome genes in microaerobic cultures was significantly higher for the *anr* strain than for the wild-type strain ($P<0.05$, Fig. 2). The higher level of *cioA* and *ccoN* expression and the increase in the respiratory activity could explain the higher ROS production in the *anr* strain, observed even in the absence of exogenous H_2O_2.

Antioxidative stress responses are displayed at low oxygen tension in the absence of Anr

Catalase activity, gluthatione content and the thioredoxin cycle are important components of antioxidative defences. Even though the *anr* mutant was more sensitive to H_2O_2 than the wild-type, catalase activity was higher in the mutant under microaerobic conditions, reaching 1440 ± 206 U (mg protein)$^{-1}$ compared with 172 ± 33 U (mg protein)$^{-1}$ for the wild-type strain. Exopolysaccharide production was also investigated due to its reported relationship with cellular protection against oxygen damage (Sabra *et al.*, 2002). The total exopolysaccharide content in aerobic cultures was similar in both strains, 10.9 ± 2.1 and 9.8 ± 3.3 g glucose (g CDW)$^{-1}$ for the wild-type and the *anr* strain, respectively ($P>0.05$). However, under microaerobic conditions the exopolysaccharide content was significantly higher for the

Table 2. Putative target genes of Anr in the *P. extremaustralis* genome related to oxidative stress

The PRODORIC software was used in the entire genome sequence.

Gene	Function	Position upstream from ATG (bp)
dnaJ	Chaperone protein	303
groEL	Chaperone protein	82
azu	Azurin	79
cbpA	Chaperone	82
cysW	Sulfate carrier, CysW	61
uspA	Universal stress protein	81
Pext1s1_16130	Protein with cold shock domains	137
clpB	Chaperone protein	150
gshB	Glutathione synthetase	79
cspD	Cold shock protein	243
fdxA	Putative ferredoxin	71
tpxB	Thiol peroxidase Tpx family	238
pipD	Peptidyl-prolyl *cis–trans* isomerase	214
bfr	Bacterioferritine	257
Pext1s1_15402	Probable thiol oxidoreductase	72

anr strain [15.2 ± 5.2 g glucose (g CDW)$^{-1}$] than for the wild-type [3.3 ± 0.3 g glucose (g CDW)$^{-1}$, $P<0.05$]. These results suggest that cells display different physiological mechanisms to cope with an oxidized state in the absence of Anr, mainly under microaerobic growth conditions.

The pool of reduced thiol molecules was estimated in microaerobic cultures through flow cytometry assays using Mercury Orange, a thiol-reactive dye that presents a preference for free reduced thiols (Thomas *et al.*, 1995). The percentage of positive cells was significantly higher in the *anr* mutant [85.9 % (78.1, 89.3)] than in the wild-type strain [61.8 % (43.6, 73.1), $P<0.05$]. However, the Gm value was similar for both strains [13.9 (12.5, 16.1) for the wild-type and 18.3 (15.4, 20.7) for the mutant strain]. When wild-type strain cultures were exposed to H_2O_2, the percentage of positive cells dropped significantly [33.1 % (26.5, 52.1) ($P<0.05$), with a Gm value of 11.4 (11.3, 11.5)]. In contrast, this drop was not observed in the *anr* strain after H_2O_2 exposure, as a similar percentage of positive cells and Gm value were observed compared with control conditions [92.1 % (76.4, 94.7) and 20.6 (15.2, 24.4)]. These results suggest an impairment in the oxidation state of the thiol pool needed for ROS detoxification in the *anr* mutant.

In silico analysis of the Anr regulon reveals several target genes related to oxidative stress

Genes related to oxidative stress responses potentially regulated by Anr were identified by using PRODORIC software in the entire genome sequence of *P. extremaustralis* (Table 2). Interestingly, several chaperones and cold shock proteins presented Anr-boxes upstream from the ATG codon. Glutathione synthase and sulfate transporter genes could be potential Anr targets and both functions are related to antioxidative mechanisms (Salunkhe *et al.*, 2005). In agreement with the results of catalase activity determinations, we could not find Anr-boxes in the promoter zone of any of the five catalase genes present in the genome of *P. extremaustralis*. In addition, a putative site of Anr regulation was found in a thiol peroxidase gene involved in the detoxification of oxidative compounds using thioredoxin molecules.

DISCUSSION

Energy metabolism and redox state maintenance are key cellular functions, crucial for bacterial adaptability and they are subjected to a complex multilayered control. In *E. coli*, several global regulatory systems, such as the ArcBA two-component system, Fnr and the global carbon storage regulatory system Csr, modulate gene expression involved in central metabolism, redox state and respiratory pathways (Nikel *et al.*, 2008; Romeo *et al.*, 1993; Tolla & Savageau, 2010). In contrast, only a few global networks related to redox homeostasis have been thoroughly analysed in *Pseudomonas* species, such as the RoxSR regulon of *P. putida* (Fernández-Piñar *et al.*, 2008). The complete repertoire of regulatory functions of other transcription factors, such as the anaerobic global regulator Anr, remains poorly understood. Anr was traditionally related to the denitrification process, pyruvate and L-arginine fermentation, and the regulation of expression of some cytochrome genes (Eschbach *et al.*, 2004; Galimand *et al.*, 1991; Ugidos *et al.*, 2008). A recent study predicted the Anr regulon in the *P. aeruginosa* genome (Trunk *et al.*, 2010).

In this work, we revealed the importance of the control exerted by Anr on cellular redox balance and oxidative stress resistance. The absence of Anr in *P. extremaustralis* at low oxygen tension provoked an oxidized state in the cells that includes a decrease in the redox ratio, a higher oxygen consumption, and an increased expression of *cioA* (CIO) and *ccoN1* (belonging to the terminal oxidase termed Cbb3-1 in *P. putida* KT2440 or Cbb3-2 in *P. aeruginosa*). In contrast, previous studies with *anr* mutants of *P. aeruginosa* and *P. putida* showed only an increase in the expression of *cioA* (Ray & Williams, 1997; Ugidos *et al.*, 2008; Kawakami *et al.*, 2010). These differences may be associated with variations in the experimental conditions used, such as differences in aeration, culture media and the presence of alternative electron acceptors. The high respiratory activity of the *P. extremaustralis anr* mutant could lead to the higher ROS production observed under conditions with restricted oxygen supply, even in the absence of exogenous oxidative agents.

It has been shown that *Pseudomonas fluorescens* reconfigures its metabolic network favouring the NADP(H) pool over NAD(H) in cells exposed to oxidative stress, mainly because several detoxification enzymes preferably use the reduced form of this dinucleotide as a cofactor (Fuhrer & Sauer, 2009; Mailloux *et al.*, 2011). In line with this notion, we detected a significantly higher NADP(H) content in the *anr* mutant in comparison with the wild-type strain, especially under conditions of microaerobiosis, in which we observed highest sensitivity to stress in the *anr* mutant. In addition, the mutant strain presented an increase in the exopolysaccharide content only under low oxygen tension conditions. Alginate production to protect cells from oxidative conditions in highly aerated cultures has been previously reported in *P. aeruginosa* (Sabra *et al.*, 2002). The alteration of these parameters [NADP(H) pool and the exopolysaccharide content] in the *anr* mutant at low oxygen tension without the addition of oxidant agents suggests a stressed cellular state. Although the redox state was affected in the *anr* mutant under aerobic conditions, the oxidative stress resistance was not altered, probably due to the presence of defence mechanisms independent of Anr.

To alleviate oxidative damage, bacteria present several antioxidative defence systems. One of the key transcription factors involved is OxyR, which activates the expression of the detoxification proteins AhpC, AhpF, AhpB, KatB, KatC and SOD (Wei *et al.*, 2012). The genome of *P. extremaustralis* exhibits a vast repertory of antioxidative

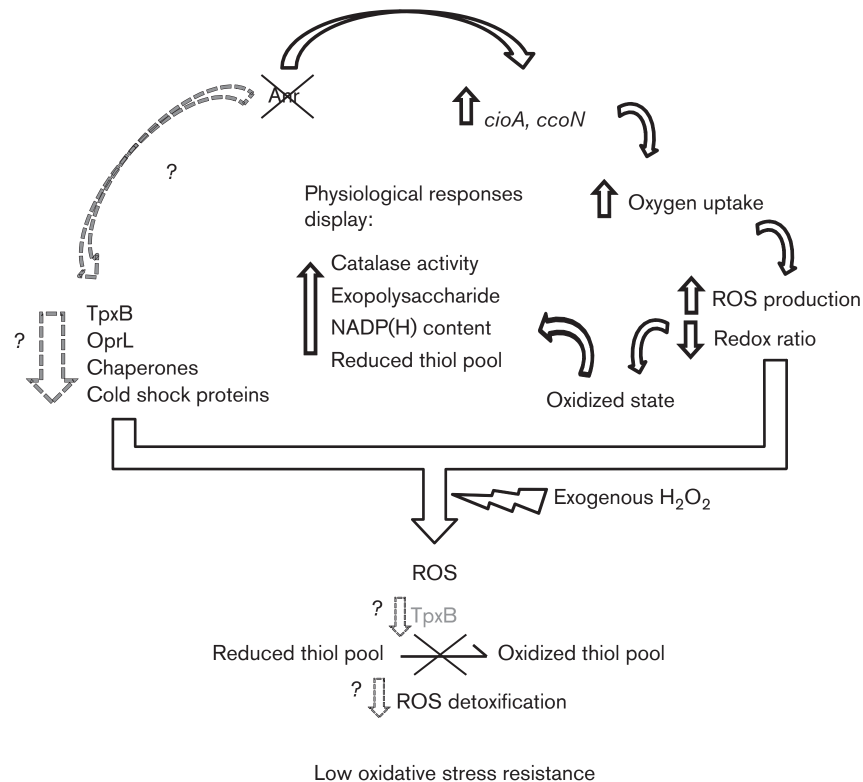

Fig. 3. Proposed model to explain the role of Anr in physiological traits of *P. extremaustralis* under microaerobic conditions. Solid black lines represent experimental data presented in this study, and grey and dashed lines with a question mark denote inferred, but not experimentally verified, processes. The absence of Anr is proposed to provoke a decrease in oxidative stress resistance. An increase in oxygen uptake and cytochrome expression in association with a higher ROS content was observed in the mutant strain. This stressful state in the *anr* mutant strain was associated with an oxidized redox state, a higher $NADPH+NADP^+$ content, a high catalase activity and exopolysaccharide production. In addition, flow cytometry assays showed an impairment in oxidation of the thiol pool in response to oxidative injury (addition of H_2O_2).

defences that includes five catalases, one superoxide dismutase, one cytochrome *c551* peroxidase, two alkyl hydroperoxide reductases and other stress-related proteins (Tribelli *et al.*, 2012). The high catalase activity and the low thiol content observed in the *P. extremaustralis anr* mutant also support the notion of an oxidized state in the cells. However, the increased catalase activity was not enough to cope with the oxidative stress under microaerobic conditions. This somewhat paradoxical result has already been observed in a *P. aeruginosa oxyR* mutant that presents high catalase activity but limited resistance to oxidative stress (Panmanee *et al.*, 2008). Gram-negative bacteria possess multiple scavenging systems to cope with oxidative stress. Among them, peroxidases, and monofunctional and bifunctional catalase–peroxidase proteins have been described (Hishinuma *et al.*, 2006; Lu *et al.*, 2008; Mishra & Imlay, 2012). It has recently been reported that monofunctional catalase activity is essential for antioxidative defence at H_2O_2 concentrations higher than 10 mM, while at lower concentrations, other mechanisms such as bifuntional catalase–peroxidase or peroxidase proteins seem to be more relevant (Horst *et al.*, 2010; Jittawuttipoka *et al.*, 2009; Mishra & Imlay, 2012; Somprasong *et al.*, 2012). However, oxidative stress responses are very complex and involve the interaction of multiple cellular components, such as transcription factors, constitutive and inducible enzymes, and other molecules that collectively alleviate oxidative damage.

By using a bioinformatics approach, we detected Anr-boxes in the promoter region of genes encoding oxidative-stress-resistance-related proteins in the *P. extremaustralis* genome. Gene coding sequences traditionally related to proper protein functioning such as chaperones, cold shock proteins and sulfate transporters presented putative Anr-boxes. These transporters are important to provide sulfur, which is necessary as an essential component of thiol groups and iron–sulfur proteins (Salunkhe *et al.*, 2005). We also detected an Anr-box in the *P. extremaustralis* OprL-coding gene. This protein seems to be important to ensure oxidative stress resistance, as a *P. aeruginosa oxyR* strain required OprL to develop a modest oxidative stress resistance (Panmanee *et al.*, 2008). It is interesting to note that none of the catalase-coding genes present in the *P. extremaustralis* genome showed putative Anr-boxes, suggesting an Anr-independent

mechanism in the transcriptional control of catalase expression.

The thiol cycle is an important detoxification mechanism that requires gluthathione and thioredoxin, as sources of reduced thiols, and the presence of specific enzymes (Carmel-Harel & Storz, 2000). In *P. extremaustralis*, the bioinformatics analysis also detected Anr-boxes in the promoter zone of genes encoding gluthatione synthase and reductase that could lead to a decrease in the glutathione pool in the *anr* mutant. However, we could not detect any Anr-boxes in genes related to thioredoxin synthesis, and thus this compound could account for the high reduced thiol pool found in the *anr* mutant.

Several bacteria have two thiol peroxidase proteins (TpxA and TpxB). TpxB, which depends on thioredoxin but not on glutathione, is essential to avoid oxidative damage in *E. coli* under anaerobic conditions (Cha *et al.*, 2004). Recently, a higher sensitivity to moderate concentrations of H_2O_2 together with a similar catalase activity has been observed in a *tpxB* mutant of *P. aeruginosa* when compared with the wild-type strain under aerobic conditions (Somprasong *et al.*, 2012), which suggest an important role of this novel system in the detoxification of moderate peroxide concentrations. We found an Anr-box upstream of the *tpxB*-coding sequence in *P. extremaustralis*. A decrease in the reduced thiol pool after exposure to H_2O_2 under microaerobic conditions was observed in the wild-type but not in the *anr* mutant strain. These results could suggest a role of Anr in the functioning of the thiol cycle for ROS detoxification under low oxygen supply. However, the participation of, and the interaction with, other cellular components cannot be ruled out.

To summarize, we were able to trace the relationship between redox homeostasis, respiratory activity and the stress defence mechanism to the Anr functions by in-depth analysis of an *anr* mutant that included the analysis of phenotypic traits as well as transcriptional patterns. Based on these results, we propose a model to illustrate the effects of Anr on these processes, which includes experimentally verified relationships as well as some potential relationships based on bioinformatic analysis and literature (Fig. 3). This work reveals the importance of Anr in the coordination of physiological processes, such as cellular redox state maintenance and oxidative stress resistance, under low oxygen conditions, adding to our current knowledge on cell physiology relevant to biotechnological set-ups.

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