The Role of PerR in O₂-Affected Gene Expression of *Clostridium acetobutylicum*^{∇†}

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In the strict anaerobe *Clostridium acetobutylicum*, a PerR-homologous protein has recently been identified as being a key repressor of a reductive machinery for the scavenging of reactive oxygen species and molecular O₂. In the absence of PerR, the full derepression of its regulon resulted in increased resistance to oxidative stress and nearly full tolerance of an aerobic environment. In the present study, the complementation of a *Bacillus subtilis* PerR mutant confirmed that the homologous protein from *C. acetobutylicum* acts as a functional peroxide sensor in vivo. Furthermore, we used a transcriptomic approach to analyze gene expression in the aerotolerant PerR mutant strain and compared it to the O₂ stimulon of wild-type *C. acetobutylicum*. The genes encoding the components of the alternative detoxification system were PerR regulated. Only few other targets of direct PerR regulation were identified, including two highly expressed genes encoding enzymes that are putatively involved in the central energy metabolism. All of them were highly induced when wild-type cells were exposed to sublethal levels of O₂. Under these conditions, *C. acetobutylicum* also activated the repair and biogenesis of DNA and Fe-S clusters as well as the transcription of a gene encoding an unknown CO dehydrogenase-like enzyme. Surprisingly few genes were downregulated when exposed to O₂, including those involved in butyrate formation. In summary, these results show that the defense of this strict anaerobe against oxidative stress is robust and by far not limited to the removal of O₂ and its reactive derivatives.

Although many bacteria are commonly classified as strict anaerobes, aerotolerance can vary greatly among these organisms. Some anaerobes even use catalase and superoxide dismutase as efficient H₂O₂- and O₂-scavenging enzymes, which were previously proposed to be limited exclusively to aerobes (45; for reviews, see references 6 and 32). Even when missing these two, fermentative anaerobes can often use their replete pool of reducing equivalents for the complete reduction of these molecules and O₂ itself. In anaerobes in particular, the level of generation of the two major reactive oxygen species (ROS), H₂O₂ and O₂^{•−}, is elevated during oxygen exposure due to their wide use of reduced flavins as cofactors in redox enzymes. Models for anaerobic oxygen detoxification via superoxide reductase, peroxidase, and NADH oxidases have been proposed for *Pyrococcus furiosus*, *Desulfovibrio vulgaris*, and, most recently, *Clostridium acetobutylicum* (33, 57, 68). These essentially include oxygen-reducing flavodiiron proteins (FDPs), superoxide-reducing desulfoferrodoxin (Dfx), and rubrerythrins as peroxidases (26, 56, 57). Reduced rubredoxin thereby acts as the intermediate electron carrier, the pool of which is continuously reloaded by an NADH-dependent rubre-

doxin-dependent oxidoreductase (55, 57). The sporulating gram-positive anaerobe *Clostridium acetobutylicum* is a classic example of a fermentative strict anaerobe. In the absence of oxygen, it ferments sugars to the organic acids acetate and butyrate or shifts to solvent formation with acetone and butanol as major products (for a review, see reference 13). Especially the latter one has regained attention lately as an attractive biofuel (14). Thus, metabolic engineering and in silico modeling of clostridial metabolism are ranked highly among recent efforts to increase productivity (39, 53, 61, 64). Furthermore, the sequenced genome and the design of microarrays for global-scale transcriptional analysis of *C. acetobutylicum* have substantially improved the understanding of solvent formation and allowed a detailed view on its unique life cycle (34, 50, 54).

During exposure to sublethal O₂ concentrations, *C. acetobutylicum* rapidly induces the expression of all components of its detoxification system and consumes dissolved O₂ (36). However, when the rate of influx exceeds the rate of consumption, the organism immediately ceases its metabolic activity and resumes only after anaerobiosis is restored (35, 51). Studies of central redox enzymes employed by anaerobes revealed that these were highly susceptible to damage of their iron sulfur sites caused directly by molecular oxygen, e.g., pyruvate-formate lyase of *Escherichia coli* or pyruvate-ferredoxin oxidoreductase (PFOR) from *C. acetobutylicum* (46, 59). *Bacteroides thetaiotaomicron*, a gram-negative anaerobe, is even employing efficient repair mechanisms to recycle this enzyme once conditions are shifted back to anaerobiosis (52).

Altogether, these observations have established the concept that anaerobes are not primarily sensitive to oxygen due to

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s) ^a	Reference or source
<i>C. acetobutylicum</i>		
MGCc15	ATCC 824 Δ CAC824I	65
Δ perR	MGCc15 Δ perR mlsR ⁺	25
<i>E. coli</i>		
pTperR _{Cac}	DH5 α plasmid pTperR _{Cac} amp ⁺	This study
<i>B. subtilis</i>		
HB1000	ZB307A attSP β	8
HB1000R _{Cac}	HB1000 amyE::perR _{Cac} cat ⁺	This study
HB0509	HB1000 perR::spc	7
HB0509R _{Cac}	HB0509 amyE::perR _{Cac} cat ⁺	This study

^a Abbreviations: amp⁺, ampicillin resistance gene; cat⁺, chloramphenicol resistance gene; spc, spectinomycin resistance gene; mlsR⁺, macrolide, lincosamide, and streptogramin B resistant.

their lack or inefficiency of scavenging enzymes. Instead, central metabolic steps in anaerobic energy conversion are poisoned by molecular oxygen (29, 31). Therefore, it does not seem surprising that some anaerobes that are temporarily but frequently exposed to oxygen found ways to avoid these bottlenecks and direct the carbon flow onto oxygen-resistant pathways (3, 52, 66). A similar strategy has been proposed for an oxygen-resistant mutant of *C. acetobutylicum* (31). Following the deletion of the clostridial perR homologue, the constitutive expression of its regulon resulted in drastically enhanced aerobic survival and permitted time-limited growth in an aerobic environment (25). In closely related facultative aerobes like *Bacillus subtilis*, the peroxide repressor PerR senses intracellular levels of H₂O₂ by metal-catalyzed histidine oxidation and controls genes for which the proteins are involved in the scavenging of hydrogen peroxide, iron storage, and DNA protection (22, 40). PerR regulation was also previously proposed for anaerobic sulfate reducers and was further supported by the coordinate induction of the predicted target genes in response to low oxygen concentrations with *Desulfovibrio vulgaris* (47, 58). The work presented here is a combined approach of microarray technology and complementational studies to obtain a global view of the organism's oxygen stimulon and refine the regulatory role of PerR in a strict anaerobe.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used in this study are listed in Table 1. *C. acetobutylicum* MGCc15 and *C. acetobutylicum* Δ perR strains were stored as spore suspensions at -20°C and grown in MS minimal medium as described previously (25). Growth was monitored as the optical density at a wavelength of 600 nm (OD₆₀₀). Cells for RNA sampling were anaerobically grown in 1-liter serum flasks, and at an OD₆₀₀ of 0.6 to 0.7, cultures were either aerated with compressed air with a rate of 0.05 liters of air min⁻¹ or left anaerobic as a control. The concentration of dissolved O₂ was measured using the WTW (Weilheim, Germany) OXI 196 oxygen meter. As a result of the continuous uptake of O₂ from the growth medium, its concentration stayed essentially below the limit of detection ($<6.25\ \mu\text{mol liter}^{-1}$). Samples for RNA isolation were taken following 1 h of O₂ exposure or from anaerobic controls at an OD of 0.6 to 0.8. *Escherichia coli* pTperR_{Cac} constitutively expressing the clostridial PerR homologue was grown overnight in 500 ml of LB medium with 100 $\mu\text{g ml}^{-1}$ of ampicillin at 30°C and 120 rpm on a rotary shaker. *B. subtilis* strains were grown at 30°C and 170 rpm in LB medium. For cultivation on solid medium, 15 g liter⁻¹ of agar was added to the medium. To select for the *B. subtilis* perR mutant strain, 100 $\mu\text{g ml}^{-1}$ spectinomycin was added as an antibi-

otic. Complemented strains carrying the clostridial perR gene were additionally selected by the addition of 10 $\mu\text{g ml}^{-1}$ chloramphenicol, and 0.5% (wt/vol) xylose was added to induce *C. acetobutylicum* perR (perR_{Cac}) expression.

Construction of plasmids. For the heterologous overexpression of the clostridial PerR protein, its gene was PCR amplified from chromosomal DNA of *C. acetobutylicum* using oligonucleotides P_TSH-CAC2634-BamHI (5'-AAAAGGATCCACGATATATCTACAA-3') and P_TSH-CAC2634-XmaI (5'-AAAACCCGGGAGCTTTATCCTTACAG-3') as primers, introducing BamHI and XmaI restriction sites (underlined). Following purification and restriction, the PCR fragment was exchanged for the hydA gene in vector pThyA, described previously by Girbal et al. (20), and the resulting vector, pTperR_{Cac}, was transformed into *E. coli* DH5 α cells. Recombinant cells were selected by the addition of ampicillin and constitutively expressed PerR from the clostridial thiolase promoter with fused Strep-tagII at the C terminus for purification (see below).

For the complementation of *B. subtilis* strains, the perR_{Cac} gene was amplified from chromosomal DNA using P_perR-pX-1 (5'-AAAAGGATTCCACGTTTT CGAAAGCAAGG-3') and P_perR-pX-2 (5'-AAAAGGATTCTTCAACTA GCAATATTG-3'), introducing BamHI restriction sites (underlined). The amplified fragments were cloned into vector pX, which integrates in the amyE locus of *B. subtilis* and allows the xylose-inducible expression of proteins (37). The recombinant vector was transformed into *B. subtilis* strains HB1000 (wild type) and HB0509 (perR mutant) using a method described previously by Cutting and Youngman (10). Positive transformants were selected due to their resistance to 10 $\mu\text{g ml}^{-1}$ of chloramphenicol.

RNA isolation and labeling. *C. acetobutylicum* cell samples for the isolation of total RNA were pelleted by centrifugation, immediately shock-frozen in liquid N₂, and stored at -70°C . RNA was isolated using a modified hot-phenol procedure described previously by Fischer et al. (17). To avoid DNA contamination, isolated RNA was digested with 20 U of RNase-free DNase (Amersham Pharmacia Biotech, Freiburg, Germany) in a total volume of 50 μl and incubated for 30 min at 37°C. The RNA was cleared from DNase by an addition of 15 μl of 2 M sodium acetate (pH 5.2), additional treatment with phenol, and precipitation with ethanol. Dried RNA was dissolved in 30 μl of H₂O, and the quality of these preparations was controlled by electrophoresis in agarose gels. The integrity of RNA was additionally controlled in a BioAnalyzer (Agilent, Böblingen, Germany) run. For the preparation of labeled cDNA, 15 μg random hexamer primer was annealed in a volume of 10 μl to 25 μg of RNA by incubation at 70°C for 10 min. Next, 1 mM dATP, dTTP, and dGTP as well as 0.4 mM dCTP, 50 μM Cy3- or Cy5-labeled dCTP (GE-Healthcare, Munich, Germany), 10 mM dithiothreitol, and 200 U SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) were added to the mixtures, and the labeling reaction mixtures with a total volume of 20 μl were incubated for 2 to 3 h at 42°C. RNA was removed from the formed heteroduplexes by the addition of 2 μl 2.5 M NaOH and incubation for 15 min at 37°C. Hydrolysis was stopped by the addition of 10 μl 2 M HEPES (pH 7.0). Labeled cDNA was separated from the reaction mixture using GFX columns (GE-Healthcare, Munich, Germany) according to the supplier's instructions, with the only modification of washing the columns four times before elution of labeled cDNA. The incorporation of Cy3 or Cy5 was checked qualitatively by a spectrophotometric wavelength scan and was quantified by using molar extinction coefficients of 150,000 liters mol⁻¹ cm⁻¹ (at 550 nm) for Cy3 and 250,000 liters mol⁻¹ cm⁻¹ (at 650 nm) for Cy5.

Microarray analysis. The *C. acetobutylicum* array was constructed by spotting 5' amino-C6-modified oligonucleotides with a length of 60 to 70 bases on CodeLink microarray slides (SurModics) using a MicroGrid II microarray spotter (Zinsser Analytic, Frankfurt, Germany). Oligonucleotides were covalently coupled to the slides surface. The array contained two identical sets of 3,840 oligonucleotides representing 99.8% of all annotated open reading frames (ORFs) in *C. acetobutylicum*. Before hybridization, samples were denatured by incubation at 98°C for 5 min. The hybridization was done using Tom Freeman hybridization buffer (18) for 15 h at 45°C with cDNA containing approximately 80 pmol of Cy3 and Cy5 in an automatic Lucidea slide processor (GE-Healthcare, Munich, Germany). Slides were washed using a program applying consecutive washes two times with 1 $\times$ SSC buffer (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% sodium dodecyl sulfate (SDS) and then with 0.1 $\times$ SSC. At the end, flushing of the hybridization chambers with isopropanol and evaporating of the isopropanol, which air dried the slides, were performed. Scanning was done using a GenePix 4000B microarray scanner (Molecular Devices, Canada) using GenePix Pro 6.0 software. Normalization was done by setting the arithmetic mean of the ratios equal to 1. Only features with fluorescence greater than the local background plus 1 standard deviation were included in the analysis. To correct for features with irregular spot morphology, only those where the ratio of medians, the ratio of means, and the regression ratio differed by less than 30% were included.

RNA was isolated from three independent cultures of *C. acetobutylicum* MGCcac15, which were either left anaerobic or exposed to air. RNA of the *perR* mutant was derived from three anaerobically growing cultures of the *C. acetobutylicum* Δ *perR* strain. RNA from *C. acetobutylicum* from anaerobic cultures was compared to RNA derived from aerated samples of the same strain and to RNA derived from anaerobic cultures of the *C. acetobutylicum* Δ *perR* strain. To avoid dye-specific effects, RNA from each sample was labeled with both dyes, and dye-swap experiments were performed. Resulting from these four technical replicates for each of the three independent cultures, a total of 12 sets of transcriptional data were obtained for each gene. The differential expression of an ORF was regarded as being significant when the average value of the ratio median from at least eight data points was either ≥ 2 or ≤ 0.5 . Full lists of transcribed genes, functional information, and regulation under O_2 or in *perR*-deleted cells are available in Tables S1 and S2 in the supplemental material. Microarray design and data are also available from the ArrayExpress database under accession no. A-MEXP-1561 (<http://www.ebi.ac.uk/microarray-as/aer/entry>). Microarray results for three genes for which transcription has not been previously identified to be either O_2 or *PerR* responsive were validated by semiquantitative reverse transcription-PCR. The expression ratios of CAC0116, CAC2459, and CAC3657 detected by reverse transcription-PCR corresponded with those obtained by microarray analysis (see Fig. S1 in the supplemental material).

Promoter analysis and motif identification. The Virtual Footprint software linked to the PRODORIC database (<http://prodoric.tu-bs.de/vfp>) (48) of gene regulation and gene expression in prokaryotes was used to identify DNA regions with a high level of similarity to the proposed regulatory motif IR2. The sequenced genome of *C. acetobutylicum* including megaplasmid pSOL1 was searched for the palindrome nucleotide sequence 5'-AATNNNTATTANNTA ATANNATT-3', with "N" representing any type of nucleotide and allowing one mismatch. The search pattern was identified in 13 different positions on the chromosome. Two sites were located in the coding region of genes and 11 sites were in the 5' noncoding region of genes within a 250-bp distance to the starting codon. Motifs with three mismatches were also identified in the upstream region of two other highly upregulated genes. A sequence logo representing a multilevel consensus sequence for each motif was achieved by an alignment of these sequences using Weblogo software (<http://www.weblogo.berkeley.edu>) (9).

Heterologous overexpression and protein purification. *PerR* from *C. acetobutylicum* was heterologously expressed as a Strep-tagII fusion protein in *E. coli* cells carrying plasmid pT_{perR}_{Cac}. Strep-tagII is an eight-residue minimal peptide sequence composed of Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (IBA GmbH, Göttingen, Germany), which binds to streptavidin and allows protein purification using streptactin affinity chromatography (60). Cells were harvested by centrifugation and washed in 50 mM Tris HCl buffer (pH 8.0) with 150 mM NaCl and 1 mM EDTA. Pellets were either stored for up to 7 days at -20°C or immediately used as a source of protein. All further purification steps for the heterologous protein used in this study were carried out as described previously (56). The protein content in the elution fractions was determined using the Bradford assay (5).

Electrophoretic mobility shift assay. A DNA fragment of the *rbr3A-rbr3B* promoter region, covering the last 235 bp of the *rbr3A* 5' untranslated region, was PCR amplified using oligonucleotides P_{3p_rbr3A} (5'-AGTGCTGCAGAAG CAGGGAAAG-3') and P_{5p_rbr3A} (5'-AATTTTCTAGATTAATCTCTCT CA-3') as primers. Reactions were carried out with *Pwo* DNA polymerase (Peglab, Erlangen, Germany) with chromosomal DNA from *C. acetobutylicum* ATCC 824 as a template. PCR products were purified from agarose gels with the Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany) and 3' digoxigenin labeled using the DIG gel shift kit (second generation; Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Labeled promoter fragments (12.5 nM) were incubated for 20 min at room temperature with 25 to 50 nM of purified *PerR*_{Cac}. Buffer contained 20 mM Tris HCl, 5% glycerol, 5 ng μl^{-1} salmon sperm DNA, 50 ng μl^{-1} bovine serum albumin, 50 mM KCl, and 100 μM MnCl₂. Unlabeled fragments were added as controls for specific binding to DNA. The reaction mixtures were applied to 1.5% (wt/vol) agarose gels with 0.5 $\times$ TBE (44.5 mM Tris-HCl [pH 8.0], 44.5 mM boric acid, 5 mM EDTA) as a running buffer. Electrophoreses were run at room temperature for 1 to 1.5 h at 65 V, and the DNA fragments were blotted onto nylon membranes (Nytran SuPerCharge nylon transfer membrane, 0.45- μm pore size; Schleicher & Schuell Bioscience, Dassel, Germany). The detection of the digoxigenin-labeled DNA was performed as described previously (44).

Zone-of-inhibition assay. To determine the sensitivity to H_2O_2 , a zone-of-inhibition assay was performed essentially as described previously by Bsat et al. (7). Fresh LB medium (50 ml) with the appropriate antibiotics was inoculated with *B. subtilis* from cultures grown overnight, and cells were grown at 30°C at 170 rpm on a rotary shaker to the mid-logarithmic growth phase (OD_{600} of 0.4

to 0.5). Aliquots of 1 ml were added to 15 ml of prewarmed (45°C) LB medium containing 1.5% (wt/vol) agar without antibiotics. The cell suspension was poured into agar plates, and after hardening, 5-mm filter paper discs containing 5 μl of 1 M H_2O_2 were placed onto the agar plates. Growth and zones of inhibition for each strain were monitored following overnight incubation at 30°C . Each strain was analyzed in triplicate. Results for each strain were reproducible within a range of $\pm 15\%$ of the maximum diameter of the inhibition zone.

Measurement of catalase activity. *B. subtilis* cells were grown to the late exponential growth phase (OD_{600} of 1.0 to 1.2) in LB medium and harvested by centrifugation. Pellets were stored at -20°C or immediately used for the preparation of crude extracts. Cells were disrupted by sonication at 4°C using the Ultrascall Desintegrator Sonopuls HD60 apparatus (Medizin und Labortechnik, Hamburg, Germany), and undisrupted cells and cell debris were removed by centrifugation at $15,000 \times g$ for 30 min. The protein concentration in the obtained cell extracts was determined with the Bradford assay (5). Catalase activity was measured spectrophotometrically at a wavelength of 240 nm. Different amounts of enzyme were added to 50 mM potassium phosphate buffer (pH 7.0) at 25°C and blanked. The reaction was started by the addition of H_2O_2 to the mixture up to a final concentration of 25 mM. The decrease in absorbance was monitored over time using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Germany). One unit of catalase activity was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 min^{-1} using an ϵ_{240} value of $43.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

RESULTS

Oxygen leads to a global transcriptional response in *C. acetobutylicum*. To identify O_2 -inducible genes, anaerobic cultures of *C. acetobutylicum* were sparged with compressed air at a rate of 0.05 liters min^{-1} liter $^{-1}$ culture volume during the logarithmic growth phase. As a result of continuous uptake, the O_2 concentration remained below detection limits ($<6.25 \mu\text{mol liter}^{-1}$), and growth continued at essentially the same rate (data not shown), which reflects previously reported results (35, 51). Samples for the analysis of global transcription were taken following 1 h of microaerobiosis during the exponential growth phase. The level of gene expression of aerated cells was compared to that of a parallel culture that was left anaerobic. Oxygen exposure drastically affected (more than threefold) the transcription of 76 genes, resulting in 60 genes which were induced and 16 genes which were downregulated. The cluster of orthologous groups (COG) classification of proteins allowed a first functional overview of the products of all genes that were differentially expressed (more than twofold) upon exposure to O_2 (67) (Fig. 1a). However, a large proportion (27% of the upregulated genes and 20% of the downregulated genes) encoded proteins that could not be grouped or were of unknown function (cluster X). The most dramatic changes occurred with genes with proteins belonging to COG clusters C (energy production and conversion; 12% upregulated and 14% downregulated genes), E (amino acid transport and metabolism; 11% and 2%, respectively), G (carbohydrate metabolism and transport; 3% and 11%, respectively), H (coenzyme transport and metabolism; 3% and 11%, respectively), and P (inorganic ion transport; 5% and 9%, respectively). Overall, these changes in global transcription suggested that *C. acetobutylicum* actively reacts to oxygen in the environment by concentrating its energy resources on the protection of essential metabolic pathways, maintenance of a reducing interior, and detoxification of ROS.

Proteins involved in detoxification and redox balance. The group of genes that were highly activated included all those which encoded the proteins previously characterized as being components of an ROS detoxification pathway or as part of an oxygen-responsive gene cluster (25, 36, 57). These include,

FIG. 1. Global transcriptional changes in wild-type *C. acetobutylicum* upon exposure to O₂ and in *C. acetobutylicum* lacking PerR. (a) The protein products of all differentially regulated genes (more than twofold) were grouped according to the COG classification scheme. Black and shaded columns indicate O₂- and PerR-responsive genes, respectively. The COG designations are as follows: C, energy; D, cell cycle control, cell division; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism and transport; I, lipid metabolism; J, translation; K, transcription and regulation; L, replication, recombination, and repair; M, cell wall and membrane proteins; N, cell motility; O, protein fate and chaperones; P, inorganic ion metabolism and transport; Q, secondary metabolites; T, signal transduction; U, secretion and intracellular trafficking; V, defense; X, poorly characterized or no cluster. (b) Venn diagrams illustrating the overlapping (gray) threefold induction (left) or threefold repression (right) of members of the oxygen stimulon (black circles) and the PerR regulon (white circles).

namely, reverse rubrerythrins (*rbr3A-rbr3B*), desulfoferredoxin (*dfx*), rubredoxin (*rd*), NADH-dependent rubredoxin oxidoreductase (NROR), and the oxygen-reducing FDPs FprA1 and FprA2 (Table 2). High levels of expression of these genes represented an efficient indicator that the global transcriptional changes observed resulted from intense oxidative stress as result of O₂ exposure. Other strongly activated genes whose products play a central role in the cell's redox balance included glutaredoxin- and thioredoxin-dependent systems (Table 2). The relatively large number of homologues and associated peroxidases suggests a vital role during oxidative stress. Interestingly, significant transcriptional changes under these conditions were not observed for the two annotated superoxide dismutases of the Cu/Zn (CAC2567) and Fe/Mn (CAC1363) types. Furthermore, the transcription of the rubrerythrins Rbr1 and Rbr2, which are characterized by their N-terminal ferritin-like domains, and the rubredoxin-like domains on the C terminus also did not respond to the presence of O₂ (Table 2).

Nucleic acid repair and iron uptake. Anaerobes in particular are highly susceptible to oxidative damage to proteins and DNA via a direct O₂-dependent inactivation of Fe-S clusters or Fenton-type chemistry (30). A more detailed view of the oxy-

gen stimulon revealed that *C. acetobutylicum* not only induces the active detoxification of oxygen and reactive derivatives but also prompts the repair of essential cellular components. The repair of damaged DNA is activated by an induced transcription of genes encoding enzymes involved in the de novo synthesis of nucleotides, e.g., enzymes of the purine metabolism (CAC1390 to CAC1395 and CAC1655) and three ribonucleotide diphosphate reductases (CAC1047, CAC3276, and CAC3277), as well as those responsible for excision and replacement (*uvrA* and *uvrB* [CAC0502 and CAC0503, respectively]) (Table 2). As expected from the conflictive role of iron as a cofactor in defense enzymes and a catalyst in Fenton chemistry, iron uptake genes were strictly regulated upon exposure to oxygen. Two Feo-type uptake systems for Fe²⁺ and one ferrichrome-dependent system were upregulated (CAC0447 and CAC0448, CAC0788 to CAC0791, and CAC1029 to CAC1032), which could support the induced Suf and Nif machinery (CAC3288 to CAC3292) in the biogenesis of Fe-S clusters. Under the same conditions, a large operon (CAC1988 to CAC1994) that is putatively involved in the uptake of Fe³⁺ and molybdenum cofactor synthesis was repressed (Table 2). Reduced iron might become scarce due to an increasing level of production of reverse rubrerythrins and FDPs, but at this point, the differential regulation of Fe²⁺ and Fe³⁺ cannot be fully explained. Previous studies with *E. coli* have shown that *moa* genes for molybdenum cofactor production are enhanced under anaerobic conditions and underlie the control of the anaerobic activator protein FNR (1, 63). Molybdenum cofactors are known to catalyze reactions of the carbon, sulfur, and nitrogen cycles and act as sites of substrate binding and reduction in nitrogenases (38, 62).

Enzymes involved in central metabolism and energy conversion. An O₂-sensitive PFOR, encoded by CAC2229 and CAC2499, is the central metabolic enzyme in *C. acetobutylicum* (46). Interestingly, the transcription of genes encoding the two subunits of an alternative α -ketoacid ferredoxin oxidoreductase (*ofrB* and *ofrA* [CAC2458 and CAC2459, respectively]) is highly induced under oxidative conditions (Table 2). The clostridial enzyme has not been studied thus far, but previously reported studies of related enzymes from the aerobic archaea *Sulfolobus* sp. strain 7 and *Aeropyrum pernix* demonstrated a wide substrate spectrum for ketoacids and, even more importantly, aerobic stability (49, 70). It is therefore likely that an oxygen-labile PFOR is replaced by an oxygen-resistant enzyme to feed the pool of acetyl coenzyme A (CoA).

The megaplasmid-carried *sol* operon (CAP0162 to CAP0164) was slightly upregulated upon exposure to O₂ (Table 2). Its protein products are thought to be involved in the early phase of solvent formation (15). Essentially all other genes whose proteins are assigned to function downstream of acetyl-CoA in the formation of butyrate are moderately downregulated (two- to fivefold) under these conditions. There are two other enzymes that are activated by O₂ and for which a central role in carbon metabolism is apparent (Table 2): one is the nonphosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GapN), catalyzing the NADP-dependent oxidation from glyceraldehyde-3-phosphate to 3-phosphoglycerate (28). Supporting its functional role during glycolysis, genes encoding the enzymes responsible for the subsequent reactions in this pathway (triosephosphate isomerase, phosphoglyceromutase, and eno-

TABLE 2. Relative transcript levels of selected genes of wild-type *C. acetobutylicum* during oxygen exposure (O₂) and of anaerobic *C. acetobutylicum* lacking PerR

ORF and function ^a	Gene	Protein function	COG class	Fold regulation		PerR box ^b
				O ₂	Lacking PerR	
Detoxification/redox balance						
CAC0327	<i>bcp</i>	Bacterioferritin comigratory protein, akyhydroperoxidase	O	5.2	16.0	+
CAC0869		Thioredoxin reductase	O	4.9	8.1	—
CAC1027	<i>fprA1</i>	Flavoprotein FprA1	C	4.4	16.1	+
CAC1363	<i>sodC</i>	Superoxide dismutase; Cu/Zn family	P		0.21	—
CAC1547	<i>trxA</i>	Thioredoxin	O	7.5		—
CAC1548	<i>trxB</i>	Thioredoxin reductase	O	10.3		—
CAC1549	<i>bsaA1</i>	Glutathione peroxidase	O	23.3		—
CAC1570	<i>bsaA2</i>	Glutathione peroxidase	O	2.6	0.1	—
CAC1571	<i>bsaA3</i>	Glutathione peroxidase	O	5.1		—
CAC2448	<i>nrOR</i>	NROR	C	7.8	16.8	+
CAC2449	<i>fprA2</i>	Flavoprotein FprA2	C	7.3	22.5	+
CAC2450	<i>dfx</i>	desulfoferrodoxin, superoxide reductase	C	18.4	29.6	+
CAC2567	<i>sod</i>	Superoxide dismutase; Fe/Mn family	P			—
CAC2575	<i>rbr1</i>	Rubrerythrin	C		0.42	—
CAC2777		Glutaredoxin	O	3.5	6.0	+
CAC2778	<i>rd</i>	Rubredoxin	C	5.2	14.4	+
CAC3018	<i>rbr2</i>	Rubrerythrin				—
CAC3306	<i>tpx</i>	Thiolperoxidase	O	3.0	3.4	+
CAC3597	<i>rbr3B</i>	Reverse rubrerythrin	C	5.6	26.9	+
Iron metabolism/transport						
CAC0447	<i>feoA1</i>	FeoA protein; involved in Fe ²⁺ transport	P	2.3		—
CAC0448	<i>feoB2</i>	Fe ²⁺ transport protein B (FeoB2)	P	2.8		—
CAC0788		Ferrichrome transport permease	P	3.3		—
CAC0789	<i>fhuB</i>	Permease	P	3.4		—
CAC0790	<i>fhuD</i>	Ferrichrome-binding periplasmic protein	P	2.6		—
CAC0791	<i>fhuC</i>	Ferrichrome ABC transporter; ATP-binding protein	P	2.3		—
CAC1029		FeoA-like protein; involved in iron transport	P	5.8		—
CAC1030		FeoA-like protein; involved in iron transport	P	8.1		—
CAC1031		FeoB-like GTPase; responsible for iron uptake	P	15.9		—
CAC1032		Predicted transcriptional regulator	K	4.3		—
CAC1988		Ferrichrome-binding periplasmic protein	P	0.27		—
CAC1989		ABC-type Fe ³⁺ transport system, ATPase component	P	0.27		—
CAC1990		ABC-type Fe ³⁺ transport system, permease component	P	0.30		—
CAC1991		Uncharacterized protein, YIIM family	S	0.32		—
CAC1992	<i>moaC</i>	Mo cofactor biosynthesis protein C	H	0.30		—
CAC1993	<i>moaA</i>	Mo cofactor biosynthesis protein A	H	0.30		—
CAC1994	<i>moaB</i>	Molybdopterin biosynthesis enzyme	H	0.32		—
CAC1995		Hypothetical protein	X	0.31		—
CAC1996		Hypothetical protein	X	0.36		—
Nucleic acid repair/metabolism						
CAC0502	<i>uvrB</i>	Excinuclease ABC subunit B	L	2.1		—
CAC0503	<i>uvrA</i>	Excinuclease ABC subunit A (ATPase)	L	4.2		—
CAC1047		Ribonucleotide-diphosphate reductase α subunit	F	9.9		—
CAC1390	<i>purE</i>	Phosphoribosylaminoimidazole carboxylase catalytic subunit	F	3.2		—
CAC1391	<i>purC</i>	Phosphoribosylaminoimidazole-succinocarboxamide synthase	F	3.1		—
CAC1392	<i>purF</i>	Amidophosphoribosyltransferase	F	2.8		—
CAC1393	<i>purM</i>	Phosphoribosylaminoimidazole synthetase	F	2.9		—
CAC1394	<i>purN</i>	Phosphoribosylglycinamide formyltransferase	F	2.8		—
CAC1395	<i>purH</i>	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	F	2.5		—
CAC1655	<i>purQ-purL</i>	Bifunctional phosphoribosylformylglycinamidine synthase	F	3.5		—
CAC1815	<i>recA</i>	Recombinase A	L	4.0		—
CAC1856		RNase HI	R	2.4	4.4	—
CAC3276	<i>nrdB</i>	Ribonucleotide-diphosphate reductase β subunit	F	13.5		—
CAC3277	<i>nrdA</i>	Ribonucleotide-diphosphate reductase α subunit	F	6.5		—
CAC3358	<i>dam</i>	Site-specific DNA methylase	L	6.4		—
CAC3499		Predicted endonuclease	G	5.3	4.1	—
Protein turnover/repair						
CAC1857		Metal-dependent peptidase	S	2.2	4.8	—
CAC3288	<i>sufC</i>	Fe-regulated ABC transporter ATPase subunit	O	2.8		—

Continued on following page

TABLE 2—Continued

ORF and function ^a	Gene	Protein function	COG class	Fold regulation		PerR box ^b
				O ₂	Lacking PerR	
CAC3289	<i>sufB1</i>	Fe-regulated ABC-type transporter membrane component	O	2.9		—
CAC3290	<i>sufB2</i>	Fe-regulated ABC-type transporter membrane component	O	3.3		—
CAC3291		Selenocysteine lyase, NifS family	E	2.6		—
CAC3292		NifU homolog involved in Fe-S cluster formation	C	2.0		—
CAC3624		6-Pyruvoyl-tetrahydropterin synthase	H	8.2		—
CAC3625		Fe-S oxidoreductase of MoaA family	O	3.1		—
Energy conversion						
CAP0162	<i>adhE1</i>	Aldehyde dehydrogenase (NAD ⁺)	C	1.9	2.2	—
CAP0163	<i>ctfA</i>	Butyrate-acetoacetate CoA-transferase subunit A	C	1.9	2.1	—
CAP0164	<i>ctfB</i>	Butyrate-acetoacetate CoA-transferase subunit B	C	2.0	2.2	—
CAC0116		CO dehydrogenase; beta chain	C	23.9	0.05	—
CAC0587		Flavodoxin	C	11.4		—
CAC0711	<i>tpi</i>	Triosephosphate isomerase	G	2.1	3.2	—
CAC0712	<i>pgm</i>	Phosphoglyceromutase	G	2.8	9.0	—
CAC0713	<i>eno</i>	Phosphopyruvate hydratase	G	3.3	6.9	—
CAC2452		Flavodoxin	C	3.6	11.6	+
CAC2458	<i>ofrB</i>	2-Oxoglutarate ferredoxin oxidoreductase, β -subunit	C	9.3	40.0	+
CAC2459	<i>ofrA</i>	2-Oxoacid ferredoxin oxidoreductase, α -subunit	C	9.1	23.8	+
CAC2499		Pyruvate ferredoxin oxidoreductase	C		0.3	—
CAC2708	<i>hbd</i>	3-Hydroxybutyryl-CoA dehydrogenase	I	0.34		—
CAC2709	<i>etfA</i>	Electron transfer flavoprotein α -subunit	C	0.29		—
CAC2710	<i>etfB</i>	Electron transfer flavoprotein β -subunit	C	0.26		—
CAC2711	<i>bcd</i>	Butyryl-CoA dehydrogenase	I	0.28		—
CAC2712	<i>crt</i>	Enoyl-CoA hydratase, crotonase	I	0.28		—
CAC2873	<i>thlA</i>	Thiolase	I	0.31		—
CAC3075	<i>buk</i>	Butyrate kinase	C	0.41		—
CAC3076	<i>ptb</i>	Phosphate acetyltransferase	C	0.35		—
CAC3657	<i>gapN</i>	NADP-GAPDH	C	19.0	26.7	+
CAC3658		Conserved membrane protein, SapB/MtgC family	S	4.0	12.5	—
CAC3659	<i>samX</i>	S-Adenosylmethionine-dependent methyltransferase	Q	2.1	4.7	—
Arginine biosynthesis						
CAC0316	<i>argF-argI</i>	Ornithine carbamoyltransferase	E		0.15	—
CAC0376		N-Dimethylarginine dimethylaminohydrolase	E	2.4	2.1	—
CAC0973	<i>argG</i>	Argininosuccinate synthase	E		0.12	—
CAC0974	<i>argH</i>	Argininosuccinate lyase	E		0.27	—
CAC2388	<i>argD</i>	N-Acetylornithine aminotransferase	E		0.11	—
CAC2389	<i>argB</i>	Acetylglutamate kinase	E		0.18	—
CAC2390	<i>argC</i>	N-Acetyl- γ -glutamyl-phosphate reductase	E		0.29	—
CAC2391	<i>argJ</i>	Ornithine acetyltransferase/N-acetylglutamate synthase	E		0.31	—

^a CAC1547 to CAC1549, CAC1570 and CAC1571, CAC2448 to CAC2450, CAC2777 and CAC2778, CAC0447 and CAC0448, CAC0788 to CAC0791, CAC1029 to CAC1032, CAC1988 to CAC1990, CAC1991 to CAC1996, CAC0502 to CAC0503, CAC1391 to CAC1395, CAC3276 and CAC3277, CAC3288 to CAC3292, CAC3624 and CAC3625, CAP0162 to CAP0164, CAC0711 to CAC0713, CAC2458 and CAC2459, CAC2708 to CAC2712, CAC3075 and CAC3076, CAC3657 to CAC3659, CAC0973 and CAC0974, and CAC2388 to CAC2391 are genes that presumably form a transcriptional unit, respectively.

^b As identified with Virtual Footprint software (48) (see Materials and Methods for details).

lase [CAC0711 to CAC0713]) are also moderately upregulated upon exposure to O₂ (Table 2). The other highly activated gene translates into a protein which belongs to the Ni-containing carbon monoxide (CO) dehydrogenase (CODH) family, which includes actual CODHs and acetyl-CoA synthases of methanogenic and acetogenic organisms (12).

The PerR regulon of *C. acetobutylicum*. A peroxide repressor-like (PerR) protein was recently shown to act as a central regulator of the O₂ detoxification system of *C. acetobutylicum*, as the full activation of its regulon triggered aerobic survival and even limited growth (25). To find targets of PerR regulation and gain insight into the gene expression profile of an aerotolerant strain, the global transcription levels in *perR*-deleted cells and cells carrying the wild-type allele were compared. The complete absence of PerR resulted in the highly

differential expression (more than threefold) of 87 genes. Of these genes, 48 were upregulated in the *perR*-deleted strain, which overlapped the oxygen stimulon of the wild-type strain. Nearly half of the genes that were induced in *perR*-deleted cells were also found to be highly expressed when the wild type was exposed to oxygen for 1 h, while the remaining 28 genes were activated exclusively in the mutant strain (Fig. 1b). All differentially expressed genes (more-than-twofold change in transcription) of the PerR mutant were also assigned to functional categories using COG. Similar to the results obtained for the O₂ stimulon, the majority of differentially expressed genes (37% of the upregulated genes and 35% of the downregulated genes) encoded proteins that either could not be grouped or were of unknown function (cluster X). Among the others, those involved in energy production and conversion (cluster C;

11% upregulated genes and 5% downregulated genes), amino acid transport and metabolism (cluster E; 7.5% and 22%, respectively), and transcription (cluster K; 9% and 5%, respectively) were highly represented in a *C. acetobutylicum* strain lacking PerR (Fig. 1a). The high number of 39 genes that were strictly downregulated (more than threefold) in the mutant strain demonstrated that PerR is essential for the expression of certain genes and/or might also have an activating role during anaerobic growth. Supporting these ideas, none of the lower-expressed genes in the mutant were found to be as lesser transcribed during oxygen exposure of the wild type (Fig. 1b). Hence, the downregulation of genes in the *perR* mutant was apparently rather independent of the cell's oxygen response. There are only two exceptions to this point, which deserve some attention. The gene encoding CODH (CAC0116) was highly activated when wild-type cells were aerated, but the transcription of this gene was most strongly repressed in the PerR mutant strain (Table 2). Similarly, the gene encoding a glutathione peroxidase (*bsaA2* [CAC1570]) was downregulated in the absence of PerR but was O₂ inducible in wild-type cells, suggesting that an inactivated state of the PerR protein might be required in order to obtain a full expression of CAC0116 and *bsaA2* upon exposure to oxygen.

PerR represses O₂ and ROS reduction. A first study of the role of PerR in *C. acetobutylicum* identified a flavodoxin (CAC2452), the FDP FprA1 (CAC1027), and, most prominently, the twin-gene-encoded reverse rubrerythrin Rbr3A-Rbr3B (CAC3598 and CAC3597) as being highly expressed in the absence of PerR (25). The transcripts of all these proteins were among the most dramatically induced ones. The highest expression values for the *perR*-deleted strain were determined for the genes and operons encoding Rbr3A-Rbr3B (CAC3598-CAC3597), FprA1 (CAC1027), glutaredoxin (CAC2777), and NROR-FprA2-Dfx (CAC2448 to CAC2450). Fluorescence counts for these transcripts exceeded those for elongation factor Tu by a factor of up to 4.3 in the case of Rbr3A-Rbr3B, which was equivalent to saturation (>80,000) when using high-resolution scans (see Table S2 in the supplemental material). Therefore, the level of induction of these highly abundant transcripts might be underestimated (Table 2). Essentially all other genes previously reported as being members of anaerobic ROS detoxification or redox balance were also activated in the mutant, e.g., rubredoxin (CAC2778), the alkylhydroperoxidase Bcp (CAC3027), and a thiolperoxidase (CAC3306). Their induction in the absence of PerR even during anaerobiosis suggested that PerR is the key regulator that represses these proteins during the anaerobic growth of the wild type. Interestingly, the glutathione and thioredoxin antioxidant system, although activated by oxygen, seems to depend on an alternative regulatory mechanism, as no significant alterations in their transcript levels were detected in the PerR mutant strain (Table 2). Similar observations were made for the genes involved in nucleic acid repair or iron metabolism and/or transport, demonstrating that PerR is not the sole level of regulation and that as-yet-unidentified regulatory circuits exist. Only few genes of the central carbon pathways were differentially expressed in the *perR* mutant. These genes include the oxoacid ferredoxin oxidoreductase operon (CAC2458 and CAC2459), GapN (CAC3657), and the enzymes that catalyze the following two glycolytic reactions.

FIG. 2. PerR consensus-like conserved sequence motif in *C. acetobutylicum*. (a) Alignment of the reversed IR2 from *C. acetobutylicum* (24) to known PerR boxes from *B. subtilis* (8) and *S. aureus* (27). (b) Sequence logo representing conserved positions in the *C. acetobutylicum* PerR box identified upstream of commonly repressed genes (Table 2).

A total of four genes were strictly repressed (>10-fold-lower expression) in the mutant strain and, hence, strongly dependent on the presence of a functional PerR protein for expression. These included two genes with a putative role in the biosynthesis or transport of siderophores (CAP0029 and CAP0030), the glutathione peroxidase BsaA2 (CAC1571), and CODH (CAC0116). Only the latter two genes were highly O₂ inducible in wild-type cells. Many genes encoding enzymes for the biosynthesis of arginine were moderately downregulated in the mutant, which suggests that arginine could function as a source of intracellular nitric oxide (NO) in *C. acetobutylicum*. Consequently, enzymes involved in the inhibition of NO production (*N*-dimethylarginine dimethylaminohydrolase [CAC0376]) and FDPs as reductive NO scavengers (26) are upregulated in PerR mutant and in O₂-exposed wild-type cells. In summary, PerR acts primarily as a repressor of an O₂-responsive, large-scale ROS defense machinery but also targets those O₂-sensitive bottlenecks that might be crucial for a continuous demand of NAD(P)H and ATP.

Direct targets of PerR repression. An earlier study of the stress-dependent transcription of reverse rubrerythrins from *C. acetobutylicum* identified an inverted repeat of 24 nucleotides (IR2) in the 5' untranslated region of *rbr3A*, for which a regulatory function was proposed (24). When reversed, this palindromic sequence shares a high level of similarity to the known consensus binding sequences of the peroxide repressor PerR from *B. subtilis* and *Staphylococcus aureus* (Fig. 2a). In the closely related facultative aerobe *B. subtilis*, PerR derepresses the transcription of multiple target genes upon its metal-catalyzed inactivation with H₂O₂ (40). To further support the idea that IR2 was the site of PerR regulation and to identify other potential target genes, a genome-wide search for IR2 was performed using Virtual Footprint software (48). When using exclusively the palindromic nucleotides and allowing a single mismatch, a total of 10 genes that carried a potential PerR box within 200 bp of their start codon were found. Nine of them were among those with the highest level of induction in the *perR* mutant, including the ones encoding FprA1, the entire O₂-responsive operon with NROR, FprA2, and Dfx, the glutaredoxin-rubredoxin operon, and both subunits of an oxoacid-ferredoxin oxidoreductase (Table 2). The O₂-induced *gapN* gene, encoding a nonphosphorylating NADP-dependent GAPDH, was also highly expressed in the

FIG. 3. Interaction of purified PerR with the *rbr3A-rbr3B* promoter region. (a) Coomassie-stained SDS-polyacrylamide gel electrophoresis (12.5%). Lanes 1 and 2, 10 and 50 μ g of crude extract from *E. coli* cells expressing PerR_{Cac}; lanes 3 and 4, purification fractions. kDa indicates the molecular mass marker. The two bands (arrows) are likely to represent two different metal-bound states of PerR as described previously for the *B. subtilis* peptide (41). (b) Electrophoretic mobility shift assays were carried out with 12.5 nM of the *rbr3A-rbr3B* promoter fragment. Fragments were incubated without protein (lane 1) or with 35 nM (lane 2) and 50 nM (lanes 3 to 5) of purified PerR. As negative controls, 25 nM (lane 4) or 50 nM (lane 5) of an unlabeled promoter fragment was also added to the reaction mixtures.

absence of PerR and preceded by a PerR box with three mismatches. Several conserved nucleotide positions were detected for this regulatory module, which could be illustrated as a sequence logo (Fig. 2b).

PerR binds to the *rbr3A-rbr3B* promoter region. To determine if the clostridial PerR homologue directly interacts with the promoter region of *rbr3A*, its gene was heterologously overexpressed in *E. coli* cells. The PerR-encoding ORF CAC2634 was cloned into plasmid pT to obtain a fusion protein with C-terminal Strep-tagII. SDS-polyacrylamide gel electrophoresis of the purified fraction yielded two bands, corresponding roughly to the calculated molecular mass of the Strep-tag fusion protein monomer of 17.1 kDa. The small size variation as well as a faint upper band might reflect different oxidation states, which were previously observed for the *B. subtilis* peptide (41) (Fig. 3a). The purified protein was incubated with a labeled DNA fragment of the *rbr3A* promoter region covering the last 235 bp of the 5' untranslated region and IR2. When these reaction mixtures were applied for gel electrophoresis, the mobility of this fragment was

FIG. 4. Zone-of-inhibition assay for different *B. subtilis* strains with H₂O₂. Mid-logarithmic-phase cells were added to prewarmed LB agar and poured into petri dishes, and a disk with 5 μ l of 1 M H₂O₂ was placed onto the agar plates. Growth and zones of inhibition for each strain were monitored following overnight incubation at 30°C. White bars indicate 10 mm. (a) HB1000 (wild type); (b) HB1000R_{Cac} (wild type expressing PerR_{Cac}); (c) HB0509 (*B. subtilis* *perR* mutant); (d) HB0509R_{Cac} (*B. subtilis* *perR* mutant expressing PerR_{Cac}).

reduced, depending on the amount of PerR in the assay (Fig. 3b, lanes 1 to 3). This shift in DNA mobility could be reversed stepwise following the addition of unlabeled fragment, giving evidence that the binding of PerR was DNA specific (Fig. 3b, lanes 4 and 5).

PerR from *C. acetobutylicum* responds to H₂O₂. The conserved binding motif and interaction with the promoter region of *rbr3A-rbr3B* suggested that the mode of PerR regulation is conserved in *C. acetobutylicum* albeit with different target genes compared to those of facultative aerobes. To monitor PerR binding in vivo and show that the PerR protein of *C. acetobutylicum* might be a peroxide sensor rather than a direct O₂ sensor, complementational studies were performed. The clostridial PerR protein was expressed from a xylose-inducible promoter in wild-type *B. subtilis* and a strain lacking a functional *perR* gene. Subsequently, their sensitivity to H₂O₂ was monitored in a zone-of-inhibition assay (Fig. 4). In the presence of xylose, the wild-type *B. subtilis* strain expressing PerR_{Cac} differed only marginally from its parent in its sensitivity to H₂O₂, which might reflect the higher affinity of *B. subtilis* PerR (PerR_{Bsu}) for its own binding site (Fig. 4a and b). *B. subtilis* cells lacking their own *perR* gene are hyperresistant to H₂O₂ due to an increased production of defense proteins (7). Following the expression of PerR_{Cac}, this hyperresistant phenotype was converted to a hypersensitive one, giving evidence that the protein from *C. acetobutylicum* can indeed acts as a repressor despite aerobic growth (Fig. 4c and d). The peroxide sensitivities of all strains were reflected largely by their catalase activities during growth in aerobic liquid cultures. Wild-type cells showed normal levels of catalase that were even moderately reduced when expressing PerR_{Cac}, but catalase was nearly 100-fold more active in the *perR* mutant strain (Fig. 5). When, in turn, the clostridial peptide was produced in the cells, catalase activity was reduced by more than

FIG. 5. Catalase activities in a PerR_{Cac} -complemented *B. subtilis* strain. Catalase was measured in crude extracts either from cells that were expressing PerR_{Cac} (white columns) or from cells of their parental strains lacking the clostridial gene (black columns). Names below the columns indicate the relevant *B. subtilis* genotypes, which were either wild type (wt) or *perR* mutants.

200-fold, to levels far below those of wild-type cells (Fig. 5). Low doses of H_2O_2 were able to partially restore catalase activity (data not shown), indicating that the key regulator of oxygen defense in *C. acetobutylicum* senses intracellular peroxide levels rather than molecular O_2 .

DISCUSSION

The finding that oxygen-sensitive enzymes are widely used as central components in the metabolism of anaerobes as well as the existence of a robust defense system has led to a refinement

of the traditional model of obligate anaerobiosis. An energy metabolism that relies exclusively on the fermentation of highly reduced substrates demands enzymes with low redox potentials and, thus, an environment that is predominantly anaerobic. *C. acetobutylicum* flourishes under fully anaerobic conditions and even maintains growth at a reduced rate when the influx of oxygen is compensated by its continuous reduction (35). Only when dissolved oxygen accumulates are central metabolic enzymes like PFOR wrecked at a rate at which metabolism is halted and viability decreases. In a natural environment, exposure to O_2 might occur gradually and, thus, allows an efficient adaptation (31). This has just recently been shown for a number of other bacteria from a diverse group of anaerobes, e.g., *Bacteroides fragilis*, *D. vulgaris*, and *Thermotoga maritima* (3, 42, 47). The experimental setup presented here was designed so that the level of influx of O_2 was low enough to ensure metabolic activity but sufficient to generate substantial cellular damage. Microaerobic growth as well as the deletion of a central regulator in oxidative stress resulted in global changes in gene expression under O_2 , leading to a preliminary model on how *C. acetobutylicum* attempts to combine O_2 /ROS removal, damage repair, and adjustments in central energy metabolism to avoid paralysis (Fig. 6).

The production of proteins involved in detoxification and cellular redox balance was most drastically activated, which was expected, as the upregulation of these components was observed recently for a number of bacteria that are considered to be anaerobes (42, 47, 66). The massive expression of all components of the proposed oxygen and ROS detoxification

FIG. 6. Model of the transcriptional response of *C. acetobutylicum* to O_2 . Differentially regulated genes are represented as encoded proteins or as functional groups and are highlighted in gray. Abbreviations used are the same as those shown in Table 2. Arrows illustrate upregulation (normal) or downregulation (blunt) at direct or indirect regulatory targets and their relative expression levels upon exposure to O_2 . Circled question marks indicate potentially activated regulatory targets of PerR . FADH_2 , reduced flavin adenine dinucleotide.

system, which involves FDPs, Dfx, NROR, rubredoxin, and reverse rubrerythrins, emphasized its vital role in survival. Consequently, all of these components were found to be the primary targets of PerR repression, as they were overexpressed in the *perR*-deleted strain and shared a common regulatory motif. Thus, it seems likely that PerR acts as a hypersensitive regulator designed to activate these genes even in the presence of traces of oxygen. The acute induction of this system in a natural environment will also support the restoration of anaerobiosis by a complete uptake of residual oxygen.

PFOR could be a major bottleneck in glucose degradation and acetyl-CoA production under microaerobic conditions. An enzyme with a higher resistance to oxidative inactivation would clearly be of advantage during oxygen exposure. At this time, induced oxoacid-ferredoxin oxidoreductase has not been functionally characterized, but the use of similar enzymes in aerobic archaea makes it tempting to speculate that a replacement occurs under these conditions (49, 70). A similar mechanism could be postulated for the nonphosphorylating GAPDH (GapN), as an increased resistance to H₂O₂ has been shown for the homologous protein from the aerotolerant anaerobe *Streptococcus mutans* (2). Interestingly, oxoacid-ferredoxin oxidoreductase and GapN seem to be two of the very few proteins that function outside the detoxification system and are still subject to direct PerR repression. This indicates their significance during the early response to oxygen, while glutathione- and thioredoxin-dependent rescue systems were PerR independent and could belong to circuits that protect from persistent exposure to oxidative conditions.

In an oxidizing environment, *C. acetobutylicum* seems to direct its electron flow toward the higher-oxidized product acetate or acetone. This is in agreement with a slightly increased level of acetate production by *C. acetobutylicum* upon exposure to O₂, which was observed during a previously reported investigation (51). The results presented here demonstrate that essentially all genes in the formation of butyrate downstream from acetyl-CoA are downregulated, suggesting that this pathway is less favorable when an oxidized dinucleotide pool is regenerated during the reduction of O₂ and ROS. At the same time, we observed a moderate upregulation of the *sol* operon, which might integrate in the larger general stress network, finally inducing solvent formation and sporulation.

Another enzyme with highly activated expression upon exposure to O₂ is a member of the CODH protein family. When the amino acid sequences of the oxygen-induced proteins from *C. acetobutylicum* were compared to the known homologues of *Carboxydotherrmus hydrogenoformans*, the clostridial peptide shows the lowest similarity to the acetyl-CoA synthase subunits (data not shown). Furthermore, the absence of genes encoding any other subunit of acetyl-CoA synthases makes it rather unlikely that this protein is involved in CO₂-dependent acetyl-CoA formation. A role for these proteins in the oxidative stress response has not been established, but a homologue from *C. hydrogenoformans* was speculated to function in ROS detoxification due to its genomic position in an operon with rubrerythrin and NROR (69). Furthermore, it cannot be excluded at this point that the clostridial protein also serves a protective function in the hydrogen production of this organism. H₂-evolving hydrogenase A1 (HyaA1) of *C. acetobutylicum* is highly sensitive to oxygen-dependent inactivation (11,

20). While low doses of molecular O₂ lead to irreversibly damaged enzymes, CO was previously shown to act as a competitive inhibitor that antagonizes O₂ and can greatly reduce the hydrogenase inactivation state (16, 43). The completely opposite expression patterns of this protein in both strains partially support this idea: the gene encoding CODH showed a high level of activation when the wild type was exposed to O₂ but the lowest level of transcription in anaerobic *perR*-deleted cells. The opposed expression of this gene in both strains addresses another interesting issue of PerR regulation. It does not seem unlikely that PerR could also function as a gene activator in its oxidized form via an as-yet-undefined mechanism. Direct gene activation by PerR was previously seen for the *B. subtilis* *sfhA* operon (21). ROS-induced transcriptional activation, although previously assigned to SoxR (OxyR), was more recently also demonstrated for the *Borellia* oxidative stress regulator BosR, a homologue of *B. subtilis* PerR (4, 23, 71).

The regulatory role in the peroxide stimulon as well as the mechanism of inactivation by H₂O₂ in a Fenton-type reaction have been thoroughly described for the *B. subtilis* peptide (7, 19, 40). In the anaerobe *C. acetobutylicum*, the external addition of H₂O₂ was not required to activate the organism's PerR regulon, while even low doses of O₂ were an adequate stimulus. The complementation of a *B. subtilis* strain lacking functional PerR with PerR_{Cac} provided evidence that the clostridial protein was a fully functional homologue of PerR in vivo. The presence of the clostridial peptide in a strain lacking PerR_{Bsu} repressed the expression of oxidative stress proteins during aerobic growth and resulted in a peroxide-hypersensitive phenotype. Low doses of external H₂O₂ inactivated PerR_{Cac} and derepressed catalase. However, as indicated from the proposed regulatory sequence motif for *C. acetobutylicum*, the level of specificity of PerR_{Cac} for the *B. subtilis* PerR boxes seems to be reduced. When PerR_{Cac} was expressed in wild-type *B. subtilis* cells, only a small increase in H₂O₂ sensitivity was determined, presumably as a result of the preferred binding of PerR_{Bsu}. Nevertheless, these data demonstrate that PerR_{Cac} could act as a peroxide sensor in vivo. Previous observations estimated that upon aeration, the intracellular H₂O₂ concentration experienced by strict anaerobes could be substantially higher than that in aerobes due to the oxidation of low-potential flavoenzymes (30). Especially for a strict anaerobe, it seems beneficial that the presence of O₂ is indirectly perceived by elevated levels of H₂O₂. The use of H₂O₂ as a signal molecule also allows the integration of metabolic activity, which determines the extent of ROS production. Consequently, *C. acetobutylicum* does not rely solely on their removal but also attempts to adapt its fermentation metabolism to lesser-reduced environments (Fig. 6). However, a broad understanding of its physiology during O₂ exposure will require further investigations, especially with respect to the biotechnological potential of this organism.

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