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The Redox-Sensitive Transcriptional Activator OxyR Regulates the Peroxide Response Regulon in the Obligate Anaerobe *Bacteroides fragilis*

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The peroxide response-inducible genes *ahpCF*, *dps*, and *katB* in the obligate anaerobe *Bacteroides fragilis* are controlled by the redox-sensitive transcriptional activator OxyR. This is the first functional oxidative stress regulator identified and characterized in anaerobic bacteria. *oxyR* and *dps* were found to be divergently transcribed, with an overlap in their respective promoter regulatory regions. *B. fragilis* OxyR and Dps proteins showed high identity to homologues from a closely related anaerobe, *Porphyromonas gingivalis*. Northern blot analysis revealed that *oxyR* was expressed as a monocistronic 1-kb mRNA and that *dps* mRNA was approximately 500 bases in length. *dps* mRNA was induced over 500-fold by oxidative stress in the parent strain and was constitutively induced in the peroxide-resistant mutant IB263. The constitutive peroxide response in strain IB263 was shown to have resulted from a missense mutation at codon 202 (GAT to GGT) of the *oxyR* gene [*oxyR*(Con)] with a predicted D202G substitution in the OxyR protein. Transcriptional fusion analysis revealed that deletion of *oxyR* abolished the induction of *ahpC* and *katB* following treatment with hydrogen peroxide or oxygen exposure. However, *dps* expression was induced approximately fourfold by oxygen exposure in Δ*oxyR* strains but not by hydrogen peroxide. This indicates that *dps* expression is also under the control of an oxygen-dependent OxyR-independent mechanism. Complementation of Δ*oxyR* mutant strains with wild-type *oxyR* and *oxyR*(Con) restored the inducible peroxide response and the constitutive response of the *ahpCF*, *katB*, and *dps* genes, respectively. However, overexpression of OxyR abolished the catalase activity but not *katB* expression, suggesting that higher levels of intracellular OxyR may be involved in other physiological processes. Analysis of *oxyR* expression in the parents and in Δ*oxyR* and overexpressing *oxyR* strains by Northern blotting and *oxyR′*::*xylB* fusions revealed that *B. fragilis* OxyR does not control its own expression.

The human intestinal obligate anaerobe *Bacteroides fragilis* possesses a complex oxidative stress response mechanism which is required to maintain extended aerotolerance compared to control cultures (24). A set of approximately 28 proteins are synthesized in response to treatment with hydrogen peroxide or oxygen exposure, but other proteins are also down regulated following a shift to aerobic conditions, and their role in the physiological adaptation to this adverse environment still remains unclear (24). The catalase gene *katB* is typical of the *B. fragilis* oxidative stress genes and is induced in mid-log phase following the addition of hydrogen peroxide or exposure to molecular oxygen or after entering the stationary phase (25). A *katB* mutant was found to be more sensitive to exogenous hydrogen peroxide under anaerobic conditions than was the parent strain, but aerotolerance in the presence of atmospheric oxygen was not significantly altered (24). The studies on resistance to peroxides led to the isolation of a KatB-overproducing mutant, IB263, with constitutive high resistance to hydrogen peroxide and organic peroxides but not atmospheric oxygen (26). Two other antioxidant proteins, AhpCF and Dps, were also constitutively expressed in the *B. fragilis* peroxide-resistant strain (26), and mutants with mutations in AhpCF were more sensitive to mutagenesis and killing by organic peroxides than was the parent strain (27). Further studies have revealed that *katB*, *ahpCF*, and *dps* are coordinately regulated at the transcriptional level, suggesting that these peroxide response genes were under the control of a common regulator (26, 27).

Recently, several other genes have been characterized as part of the oxidative stress response in *B. fragilis*, but these were not part of the peroxide regulon. The genes encoding ribonucleotide diphosphate reductase, *nrdA*, a pyridoxal 5′-phosphate binding protein, *oip-1*, and superoxide dismutase, *sod*, were induced by a peroxide-independent oxygen-dependent mode, whereas *recA* and malonyl coenzyme A-acyl carrier protein transacylase mRNAs were down regulated following an oxidative stress insult in *B. fragilis* (D. J. Smalley, E. R. Rocha, and C. J. Smith, Abstr. 97th Gen. Meet. Am. Soc. Microbiol. 1997, abstr. k-141, p. 365, 1997). Thus, these studies confirm that the physiological response of the anaerobe *B. fragilis* to oxidative stress is not a simple adaptation to an adverse environment but that instead there are multiple regulatory mechanisms that control specific aspects of the response.

Similarly, the peroxide and superoxide stress responses in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium are independent, and numerous studies have shown that they are controlled at the transcriptional level by two major regulators, OxyR, and SoxRS, respectively (34, 35). In contrast, very little is known about how anaerobic bacteria control the expression of genes involved in the oxidative stress response, and no regulatory genes have been found. Thus, based on the experimental evidence for the presence of oxidative stress regulators in *B. fragilis* mentioned above, we used the peroxide-resistant strain as a genetic tool to identify the mechanism controlling the peroxide response in *B. fragilis*. In this paper we report on the identification and characterization of an OxyR

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TABLE 1. Relevant characteristics of *B. fragilis* strains and plasmids used in this study

Strain or plasmid	Phenotype[a]	Reference
Strains		
638R	Clinical isolate, Rif	23
IB263	638R hydrogen peroxide-resistant *oxyR*(Con) Rif	27
IB272	638R *katB′*::*xylB bglA* Rif Erm	Smalley et al., abstract
IB277	638R *ahpC′*::*xylB* Rif Erm	27
IB278	IB263 *ahpC′*::*xylB* Rif Erm	27
IB294	638R *oxyR′*::*xylB bglA* Rif Erm	This study
IB295	638R *dps′*::*xylB bglA* Rif Erm	This study
IB296	IB263 *oxyR′*::*xylB bglA* Rif Erm	This study
IB297	IB263 *dps′*::*xylB bglA* Rif Erm	This study
IB298	638R Δ*oxyR*::*tetQ* Rif Tet	This study
IB299	IB263 Δ*oxyR*(Con)::*tetQ* Rif Tet	This study
IB300	638R Δ*oxyR*::*tetQ ahpC′*::*xylB* Rif Erm Tet	This study
IB301	IB263 Δ*oxyR*(Con)::*tetQ ahpC′*::*xylB* Rif Erm Tet	This study
IB307	638R Δ*oxyR*::*tetQ oxyR′*::*xylB bglA* Rif Erm Tet	This study
IB308	IB263 Δ*oxyR*(Con)::*tetQ oxyR′*::*xylB bglA* Rif Erm Tet	This study
IB309	638R Δ*oxyR*::*tetQ dps′*::*xylB bglA* Rif Erm Tet	This study
IB310	IB263 Δ*oxyR*(Con)::*tetQ dps′*::*xylB bglA* Rif Erm Tet	This study
Plasmids		
pFD288	Shuttle vector, *oriT*, pUC19::pBI143 chimera, (Sp) Erm	33
pFD516	Suicide vector, derived from deletion of pBI143 in pFD288 (TetX) (Sp) Erm	33
pFD697	Reporter gene vector, 1.2-kb *Eco*RI fragment with promoterless β-xylosidase/arabinosidase (*xylB*) cloned into *Eco*RI site of pFD516 with a 600-bp *Taq*I internal fragment of *bglA* in the *Cla*I site, (Sp) Erm	Smalley et al., abstract
pFD770	1.34-kb *oxyR*(Con) fragment cloned into the *Sma*I site of pFD288 containing a 2.4-kb blunted *Bam*HI-*Nar*I *cfxA* fragment into the blunted *Eco*RI-*Nar*I sites, (Sp) Erm Cfx	This study
pFD772	1.34-kb *oxyR* fragment cloned into the *Sma*I site of pFD288 containing a 2.4-kb blunted *Bam*HI-*Nar*I *cfxA* fragment cloned into the blunted *Eco*RI-*Nar*I sites, (Sp) Erm Cfx	This study

[a] Erm, erythromycin resistance; Cfx, cefoxithine resistance; Rif, rifampin resistance; Tet, tetracycline resistance; Sp, spectinomycin resistance. Parentheses indicate antibiotic resistance expression in *E. coli*.

homologue and show that a mutated *oxyR* gene is responsible for the constitutive expression of the peroxide response in the peroxide-resistant mutant.

MATERIALS AND METHODS

Strains and growth conditions. The *B. fragilis* strains and plasmids used in this study are listed in Table 1. All strains were grown anaerobically in brain heart infusion broth supplemented with hemin, cysteine, and $NaHCO_3$ (BHIS) for routine cultures and genetic procedures (32). Cysteine was omitted in some experiments where indicated, and 20 μg of rifampin per ml, 50 μg of gentamicin per ml, 5 μg of tetracycline per ml, 10 μg of erythromycin per ml, and/or 25 μg of cefoxitin per ml were added to the medium when required.

Cloning and DNA sequencing of *oxyR*. All DNA modifications and manipulations were carried out by standard methods (4, 28). In an effort to amplify *oxyR* homologues from the *B. fragilis* chromosome, oligonucleotide primers were designed based on conserved amino acid sequences adjacent to the DNA binding motif MNIR(Q)D(Q)LE(K)YL(I)V(A)A and the functional cysteine residue conserved-region consensus E(D)E(D)GHCL(F)RD(N)Q of bacterial OxyR proteins available in the database. The sense and antisense oligonucleotide sequences are 5′-ATG AAY ATH MRI SAI YTI RAR TAY HTI GYI GC and 5′-TGR TRI CKI ARR CAR TGI CCI TCI TC, respectively. A 600-bp fragment was then amplified by *Taq* polymerase using a PCR amplification kit (Qiagen, Valencia, Calif.). The thermocycling conditions were set using touchdown annealing temperatures as follows: 5 cycles at 50°C, 5 cycles at 45°C, 5 cycles at 45°C, and 25 cycles at 35°C. The denaturing and extension temperatures for all reaction cycles were set at 94°C for 15 s and 72°C for 1 min, respectively. The amplified fragment was extracted from an agarose gel, ligated into cloning vector pGEM-T (Promega, Madison, Wis.), and electrotransformed into *E. coli* DH10B, resulting in pFD726. Southern blot hybridization analysis using the cloned fragment as a probe revealed homology to 2.5-kb *Eco*RV and 5-kb *Pst*I DNA fragments in the *B. fragilis* chromosome. Then, using inverse PCR (13), the 2.5-kb *Eco*RV and 5-kb *Pst*I fragments were amplified by Platinum *Taq* High Fidelity DNA polymerase (Life Technologies, Rockville, Md.) using the specific oligonucleotide primers 5′-CGG TAA CAC TGC CAA TCG GAA TG and 5′-GCT GGA TGA TGC CGC ATT AAC GG, based on known sequences. The amplified fragments were then cloned into the pGEM-T vector for further nucleotide sequencing. The procedure to isolate the *oxyR* gene from the peroxide-resistant strain IB263 was carried out by inverse PCR as above using the IB263 chromosome as template. Automated nucleotide sequencing was performed on double-stranded DNA templates (Molecular Biology Resource Facility, University of Tennessee, Knoxville, Tenn.). Additional oligonucleotide primers were designed based on available sequence information to extend and confirm the existing sequence.

RNA extraction, Northern blot hybridization, and primer extension. Total RNA extraction and Northern blot analysis of mRNA were carried out as previously described (25). Internal fragments of *dps* and *oxyR* were used as specific probes. Densitometry analysis of the autoradiograph was normalized to the relative intensity of total 23S and 16S rRNA detected on the ethidium bromide-stained agarose gel to correct for any loading differences.

Primer extension analysis was performed on total RNA obtained from mid-log-phase cells of *B. fragilis* 638R and IB263 grown anaerobically and then subjected to oxidative stress conditions as described previously (25). A *dps*-specific oligonucleotide, 5′-GAT GTT CCA GTG AAA TCC TCT CAG GTT TGC, complementary to nucleotides 97 to 137 of the *dps* coding region and an *oxyR*-specific oligonucleotide, 5′-CAG TTT CAC CCC CAA TTC GTC TTC CAG CTT CTG G, complementary to nucleotides 108 to 141 of the *oxyR* coding region were labeled with [γ-^{32}P]ATP and used as primers for the reverse transcriptase reaction as described previously (25). The extended labeled product was electrophoresed on 8% polyacrylamide gels containing urea. A nucleotide sequence ladder was prepared with Sequenase (USB, Cleveland, Ohio) using a template covering the transcription start site region with the same oligonucleotides that were used for the reverse transcription reactions.

Construction of *oxyR* deletion mutants. Briefly, a 2.7-kb chromosome fragment containing the *oxyR* region was amplified by PCR with two oligonucleotides containing nucleotide modifications to create sites for *Eco*RI and *Bam*HI. The amplified fragment was then cloned into the *Eco*RI and *Bam*HI sites of the suicide vector pFD516 (33) to create pFD750. Subsequently, an internal 652-bp *Sal*I-*Nde*I (blunted) fragment from the *oxyR* gene was removed and replace with a *Sal*I-*Sma*I *tetQ* fragment to construct pFD754 (Δ*oxyR*::*tetQ*). pFD754 was mobilized from *E. coli* DH10B into both *B. fragilis* 638R and IB263 strains by triparental matings (31), and exconjugants were selected on BHIS agar plates containing 20 μg of rifampin per ml, 100 μg of gentamicin per ml, and 5 μg of tetracycline per ml. Determination of sensitivity to erythromycin and Southern blot analysis of chromosomal DNA were carried out to confirm the double-crossover genetic allele exchange of pFD754 into the *B. fragilis* chromosome to create the *oxyR* deletion mutants 638R Δ*oxyR*::*tetQ* (IB298) and IB263 Δ*oxyR*::*tetQ* (IB299).

Construction of *oxyR′* and *dps′* β-xylosidase (*xylB*) transcriptional fusions. A 187-bp *Dra*I-*Hinc*II fragment from pFD750 was cloned into the *Sma*I site of pUC19 in both orientations. Then *Sph*I-*Sst*I fragments from both constructs were cloned into the *Sph*I-*Sst*I sites of pFD700 containing a 600-bp fragment from *B. fragilis bglA* as a target for integration into the *B. fragilis* chromosome (Smalley et al., Abstr. 97th ASM Meet.). A 1.2-kb *Eco*RI fragment from pXA1 containing the promoterless xylosidase/arabinosidase (*xylB*) bifunctional reporter gene (42) was cloned into the unique *Eco*RI site of the new construct. Restriction analysis were used to confirm the orientation of the new constructs, pFD752 and pFD753, containing the *oxyR′*::*xylB* and *dps′*::*xylB* transcriptional fusions, respectively. pFD752 and pFD753 were mobilized from *E. coli* DH10B into *B. fragilis* strains by triparental matings, and they integrated into the *bglA* gene.

Enzyme assays. β-Xylosidase and catalase activity assays were carried out in bacterial crude extracts as described previously (27). Cell crude extracts were obtained from mid-log-phase anaerobic cultures of *B. fragilis* in BHIS without cysteine supplementation. The cultures were treated with 50 μM hydrogen peroxide for 15 min or by exposure to atmospheric oxygen for 1 h as described previously (24).

Complementation of *oxyR* mutants with *oxyR* and *oxyR*(Con). A 1.34-kb *oxyR* fragment was amplified by PCR with Platinum *Taq* High Fidelity DNA polymerase from the 638R and IB263 chromosomes. The *oxyR*(Con) and *oxyR* fragments were cloned into the *Sma*I site of shuttle vector pFD288 (33). Also, a 2.4-kb blunted *Bam*HI-*Nar*I DNA fragment containing a cefoxitin (*cfxA*) cassette was cloned into the blunted *Eco*RI-*Nar*I sites to produce pFD770[*oxyR*(Con)] and pFD772(*oxyR*), respectively. pFD770 and pFD772 were mobilized into *B. fragilis* strains by triparental matings. Transconjugants were selected on BHIS agar plates containing 20 μg of rifampin per ml, 100 μg of gentamicin per ml, 10 μg of erythromycin per ml, and 25 μg of cefoxitin per ml.

DNA sequence analysis and database comparison. Computer analysis of nucleotide and amino acid sequence data was performed using the University of Wisconsin Genetics Computer Group DNA sequence analysis software (version 10) (11). Phylogenetic relationships were inferred by the parsimony method with the PHYLIP phylogeny inference package (version 3.5) (14) from a multiple amino acid sequence alignment generated by Pileup. A consensus tree was constructed from 100 bootstrap replications.

Other gene sequences (and their products) used for the analysis, together with their respective GenBank accession numbers, are as follows: *Bacillus subtilis* metalloregulation DNA-binding protein MrgA (P37960), *B. subtilis* general stress protein GSP20U (P80879), *Borrelia burgdoferi* neutrophil-activating protein A (NapA) (AE001169), *E. coli* OxyR (P11721), *E. coli* Dps (P27430), *Erwinia chrysanthemi* OxyR (AJ005255), *Haemophilus influenzae* OxyR (P44418) and *H. influenzae* hypothetical protein HI1349 (P45173), *Helycobacter pylori* neutrophil-activating protein A (NapA) (U16121), *Listeria innocua* non-heme-iron-containing ferritin (P80725), *Mycobacterium leprae* OxyR (P52678), *Rickettsia prowazekii* unknown protein (AJ235273), *Streptococcus pneumoniae* unknown protein (AF055720), *Synechocystis* strain PCC6803 hypothetical 17.8-kDa protein YI94 (P73321), and *Xanthomonas campestris* pv. phaseoli OxyR (U94336). *Porphyromonas gingivalis* OxyR and Dps preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. *Bordetella pertussis* OxyR and *Neisseria meningitidis* OxyR preliminary sequence data were obtained from the Sequencing Group at Sanger Center. *Pseudomonas aeruginosa* OxyR preliminary sequence data were obtained from the Pseudomonas Genome Project.

Nucleotide sequence accession numbers. The nucleotide sequences of the *B. fragilis* 638R *oxyR* and *dps* genes and the IB263 *oxyR*(Con) gene have been deposited in GenBank under accession numbers AF206033 and AF206034, respectively.

RESULTS

Analysis of the *oxyR* and *dps* nucleotide sequences. Previous work on the regulation of *katB* (25) and *ahpCF* (27) and the phenotype of a hydrogen peroxide-resistant mutant (26) suggested that there was coordinate regulation of at least some oxidative stress genes in *B. fragilis*. The possibility that this control was mediated by OxyR was strengthened by the observation of an *oxyR* homologue in the genome sequence of a closely related anaerobe, *P. gingivalis*. Thus, using a PCR approach with primers based on the conserved regions of all known OxyR proteins (described in Materials and Methods), we cloned the *B. fragilis* 638R *oxyR* gene. This gene is composed of an open reading frame containing 927 nucleotides, and the deduced amino acid sequence revealed a 308-amino-acid peptide with significant homology to OxyR and other members of the LysR-type family of transcriptional activators in the databases. As expected, *B. fragilis* OxyR had the highest homology (58.6% identity and 66% similarity) to a hypothetical OxyR found in *P. gingivalis*. However, compared to other facultative and aerobic organisms, this similarity was greatly reduced to about 40% identity. The alignment of OxyR amino acid sequences is shown in Fig. 1A. The helix-turn-helix motif region for DNA binding and promoter recognition present at the N-terminal domain of LysR-type regulators (19, 29) and the functional cysteine residues (C199 and C208) essential for the redox activity in *E. coli* OxyR (18, 45) are highly conserved in *B. fragilis* OxyR (Fig. 1A).

The phylogenetic relationship of 22 bacterial OxyR and 5 members of the LysR-type family of transcriptional regulators was determined from a progressive multiple alignment of the amino acid sequences followed by parsimony analysis (data not shown). This comparison clearly shows that the obligate anaerobes *B. fragilis* and *P. gingivalis* were clustered in a branch separated from other gram-negative eubacteria.

When the nucleotide sequence upstream of *oxyR* was analyzed, it revealed an ORF containing 474 nucleotides oriented in the opposite direction from *oxyR* translational start codon (Fig. 1B and 2). The deduced amino acid sequence revealed a peptide with homology to Dps, a bacterial oxidative stress and stationary-phase nonspecific DNA binding protein (1, 2). The first 30 N-terminal amino acids showed 100% identity to the N-terminal sequence previously obtained by Edman degradation of a protein in the peroxide-resistant mutant IB263 (26). This therefore confirms the presence of a *dps* gene in *B. fragilis* and the expression of its respective product. Alignment of the *B. fragilis* Dps with other Dps homologues revealed high homology to the putative *P. gingivalis* Dps (47.5% identity, 60% similarity) and the putative *H. influenzae* Dps (45% identity, 56% similarity). The alignment of the *B. fragilis* Dps sequence with other bacterial Dps homologues revealed the presence of the carboxylated amino acids presumed to be involved in iron withholding in *L. innocua* Ftn and *H. pylori* NapA (6, 39) and the DNA binding motif (Fig. 1B). The phylogenetic relationship between *B. fragilis* Dps and 19 bacterial Dps homologues was determined from a progressive multiple alignment of the amino acid sequences followed by parsimony analysis. Interestingly, *B. fragilis* Dps was clustered with *H. influenzae* and *P. gingivalis* Dps in a branch also containing *B. burgdoferi* NapA (data not shown).

Identification of an *oxyR* mutation in the constitutive peroxide-resistant mutant IB263. To investigate whether the constitutive peroxide-resistant strain IB263 had an altered OxyR regulator, the entire IB263 *oxyR* operon was sequenced. Analysis of the nucleotide sequence revealed a single-base substitution (A to G) at codon 202 (GAT to GGT), leading to a D202G amino acid substitution in the IB263 OxyR protein compared to the parent. No other base substitution was found in the promoter and coding regions of *oxyR* (data not shown). To confirm this point mutation, three independent PCR amplifications of the IB263 and 638R chromosomal *oxyR* regions were performed using High Fidelity proofreading *Taq* polymerase. All amplified IB263 *oxyR* nucleotide sequences obtained showed the same single-nucleotide base substitution at codon 202 compared to 638R *oxyR* sequences. This confirms that a point mutation had occurred in the IB263 *oxyR* gene, which is hereafter named *oxyR*(Con). D202 is in a highly conserved region around the functional cysteine C199 region at the C-terminal domain of the OxyR protein.

Regulation of *dps* and *oxyR* mRNA expression by oxidative stress. To investigate the expression of *dps* and *oxyR*, total RNA extracted from mid-log-phase cells exposed to different oxidative stress conditions was probed with specific internal DNA fragments. Northern blot hybridization analysis revealed that expression of *dps* mRNA was regulated at the transcrip-

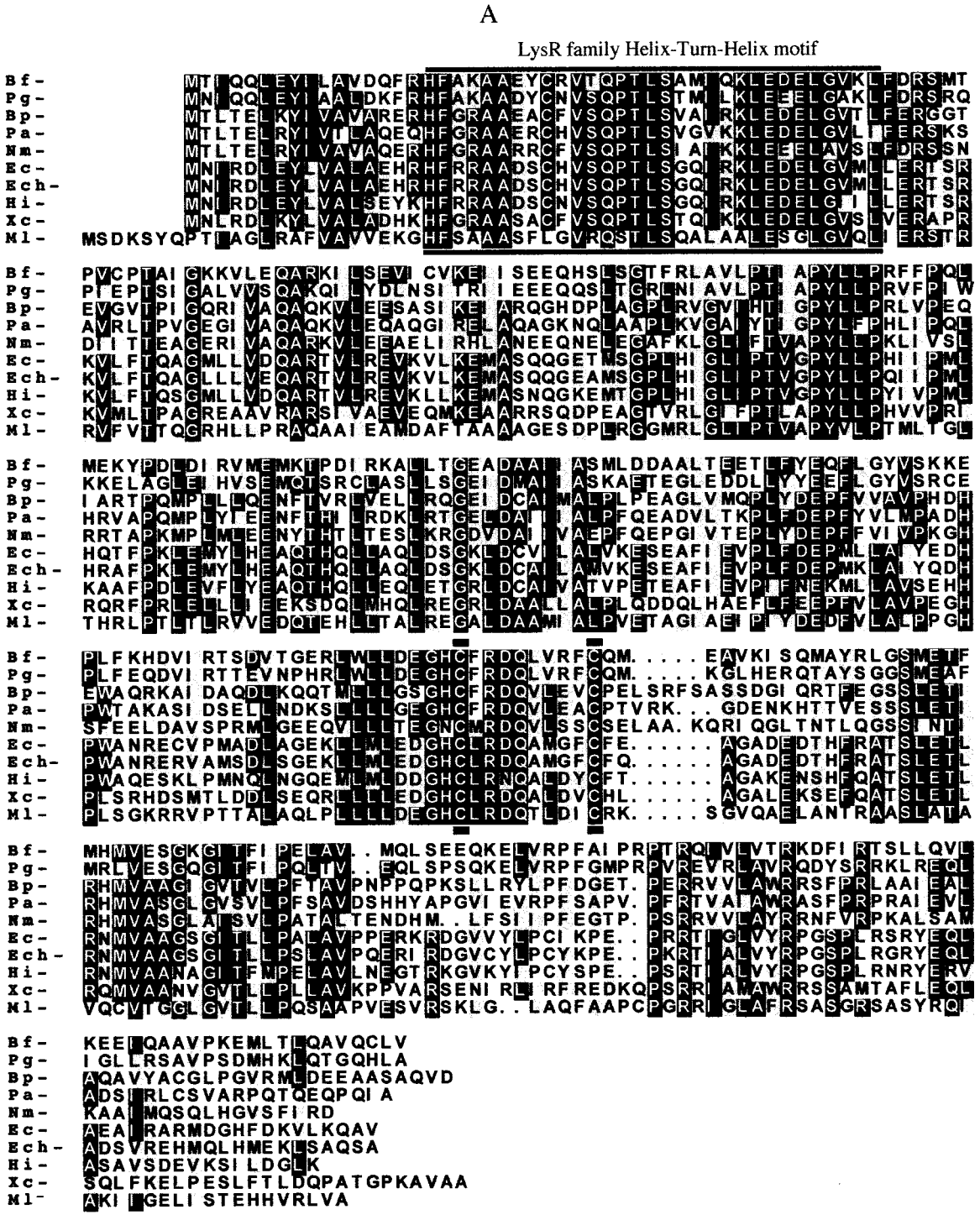

FIG. 1. Multiple alignment of the *B. fragilis* (Bf) deduced amino acid sequences for OxyR (A) and Dps (B) with other bacterial OxyR and Dps homologue amino acid sequences, respectively. *E. coli* (Ec), *B. burgdorferi* (Bb), *B. pertussis* (Bp), *B. subtilis* (Bs), *E. chrysanthemi* (Ech), *H. influenzae* (Hi), *H. pylori* (Hp), *L. innocua* (Li), *M. leprae* (Ml), *N. miningitis* (Nm), *P. gingivalis* (Pg), *P. aeruginosa* (Pa), *R. prowazekii* (Rp), *S. pneumoniae* (Sp), *Synechocystis* sp. (Sy), and *Xanthomonas campestris* (Xc) sequences were used. Lines drawn above and below the amino acid sequences indicate the LyR helix-turn-helix DNA-binding nucleotide sequence motif and functional redox-active cysteine residues of *E. coli* OxyR C199 and C208 (29, 45) in panel A and the Dps protein signature consensus pattern associated with DNA binding properties (6, 43) in panel B. Consensus of at least 50% identical amino acid residues are labeled with black boxes. Conserved amino acid substitutions are depicted by grey boxes. The respective protein descriptions and GenBank accession numbers for the sequences are listed in Materials and Methods.

tional level. Transcripts of approximately 0.5 kb were detected, suggesting that *dps* was transcribed as a monocistronic mRNA (Fig. 3A). Densitometric analysis of the Northern blots showed approximately a 500-fold increase in the level of *dps* mRNA in cultures treated with H_2O_2 or exposed to oxygen compared to that in anaerobic cultures. *dps* mRNA was constitutively expressed anaerobically (250-fold increase) in IB263 compared to anaerobic cultures of the parent 638R (Fig. 3A). When

B

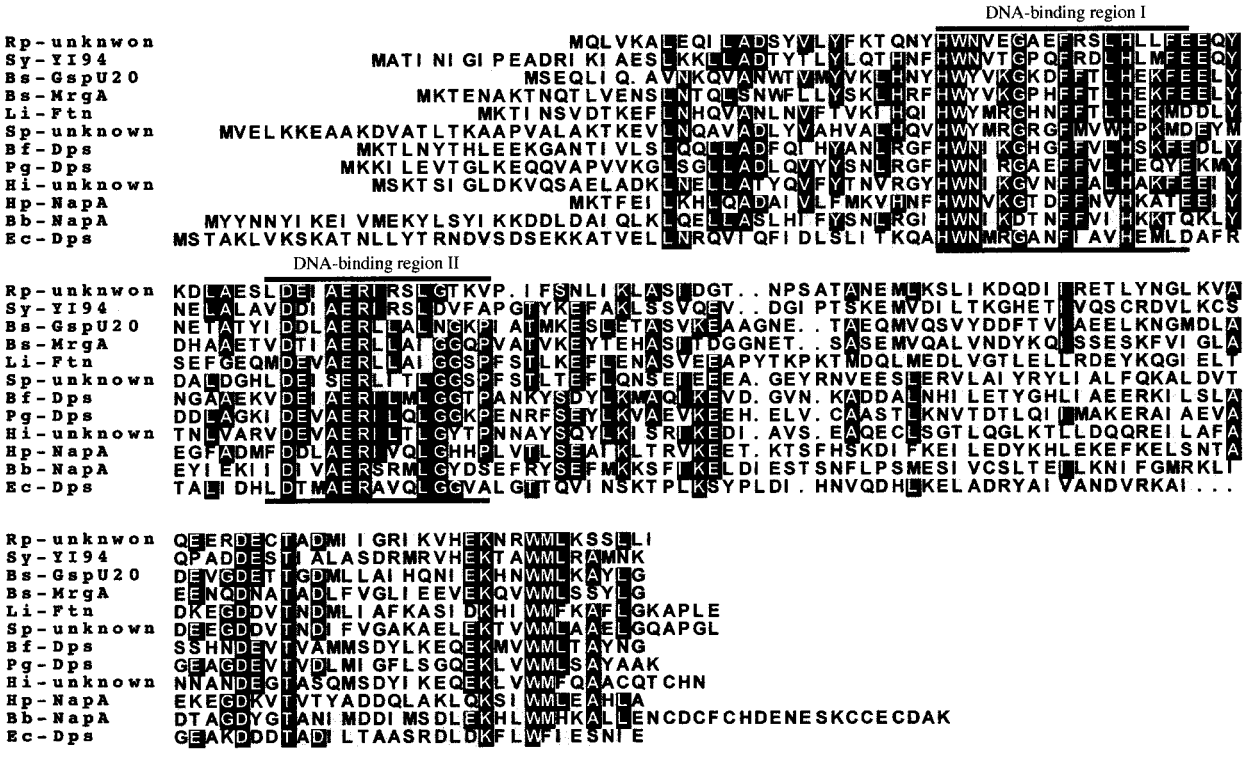

FIG. 1—*Continued.*

Northern blots were probed with the *oxyR* fragment, the autoradiographs revealed an mRNA of approximately 1.0 kb, suggesting that *oxyR* was also transcribed as a monocistronic *oxyR* mRNA (Fig. 3C). In contrast to *dps*, *oxyR* mRNA levels were not significantly altered after treatment with hydrogen peroxide and exposure to oxygen in the parent or the *oxyR*(Con) mutant strain.

Primer extension analysis of *dps* and *oxyR* mRNAs showed that *dps* mRNA starts at a cytosine nucleotide 49 bp upstream of the *dps* translation start codon whereas *oxyR* mRNA starts at guanine nucleotide 34 bp upstream of the *oxyR* translation start codon in the opposite strand. The *dps* and *oxyR* intergenic region was 142 nucleotides in length, and the predicted −10 and −35 promoter regions for both genes were found overlapped. A diagram of the *dps* and *oxyR* −10 and −35 promoter regions and transcription start nucleotides is shown in Fig. 2. These findings indicate that the nonspecific DNA binding *dps* is strongly upregulated by oxidative stress while *oxyR* transcription levels are not altered following oxidative stress.

***oxyR*-dependent control of the oxidative stress response genes *katB*, *ahpCF*, and *dps*.** To investigate the role of OxyR in expression of the peroxide regulon, *oxyR* deletion mutants of the parent strain and the hydrogen peroxide-resistant mutant (IB263) were constructed by double-crossover allelic gene exchange. Preliminary characterization of the mutants showed that while they were highly sensitive to hydrogen peroxide killing, their aerotolerance was just marginally decreased, as indicated by viable-cell counts (data not shown). The effect of the *oxyR* deletion on gene expression as measured by analysis of β-xylosidase transcriptional fusions and catalase activity following oxidative stress is shown in Fig. 4. The induction of *katB* and *ahpCF* by both oxygen and hydrogen peroxide was nearly abolished in *oxyR* mutants compared to the parent strains. This indicates that a functional OxyR is essential for induction of these stress response proteins (Fig. 4A and C). Moreover, it also confirms that the *oxyR*(Con) is responsible for the constitutive peroxide response phenotype in IB263. In contrast to *ahpCF* and *katB*, *dps* expression was still significantly induced (fourfold) following oxygen exposure in both 638R Δ*oxyR* and IB263 Δ*oxyR*, suggesting that *dps* may be under dual regulation by OxyR and an oxygen-dependent, OxyR-independent mechanism (Fig. 4B). In addition, *dps* expression under anaerobic conditions was not altered in the Δ*oxyR* mutants, indicating that there may also be a growth-dependent regulation. The levels of *oxyR* expression were not affected by deletion of the *oxyR* gene from the parent strains, as determined by using the transcriptional reporter fusion *oxyR′*::*xylB* (Fig. 4D).

Genetic complementation of *oxyR* in Δ*oxyR* mutants with pFD770[*oxyR*(Con)] and pFD772(*oxyR*). Restoration of the OxyR phenotype in Δ*oxyR* strains was investigated by complementation with plasmids pFD770[*oxyR*(Con)] and pFD772 (*oxyR*) (Fig. 5). The copy number of these plasmid constructs is estimated at 15 to 20 copies per cell based on the parent replicon pIB143 (33). Considering the lack of *oxyR* autoregulation, this would suggest that there was a 15 to 20-fold overexpression of these genes during the complementation experiments. The presence of the wild-type *oxyR* gene restored induction of *ahpCF* and *dps* expression during treatment with hydrogen peroxide or oxygen exposure, while complementation of Δ*oxyR* with *oxyR*(Con) restored the constitutive regulation of *ahpCF* and *dps* expression compared to anaerobic culture controls (Fig. 5A and B). These findings establish that

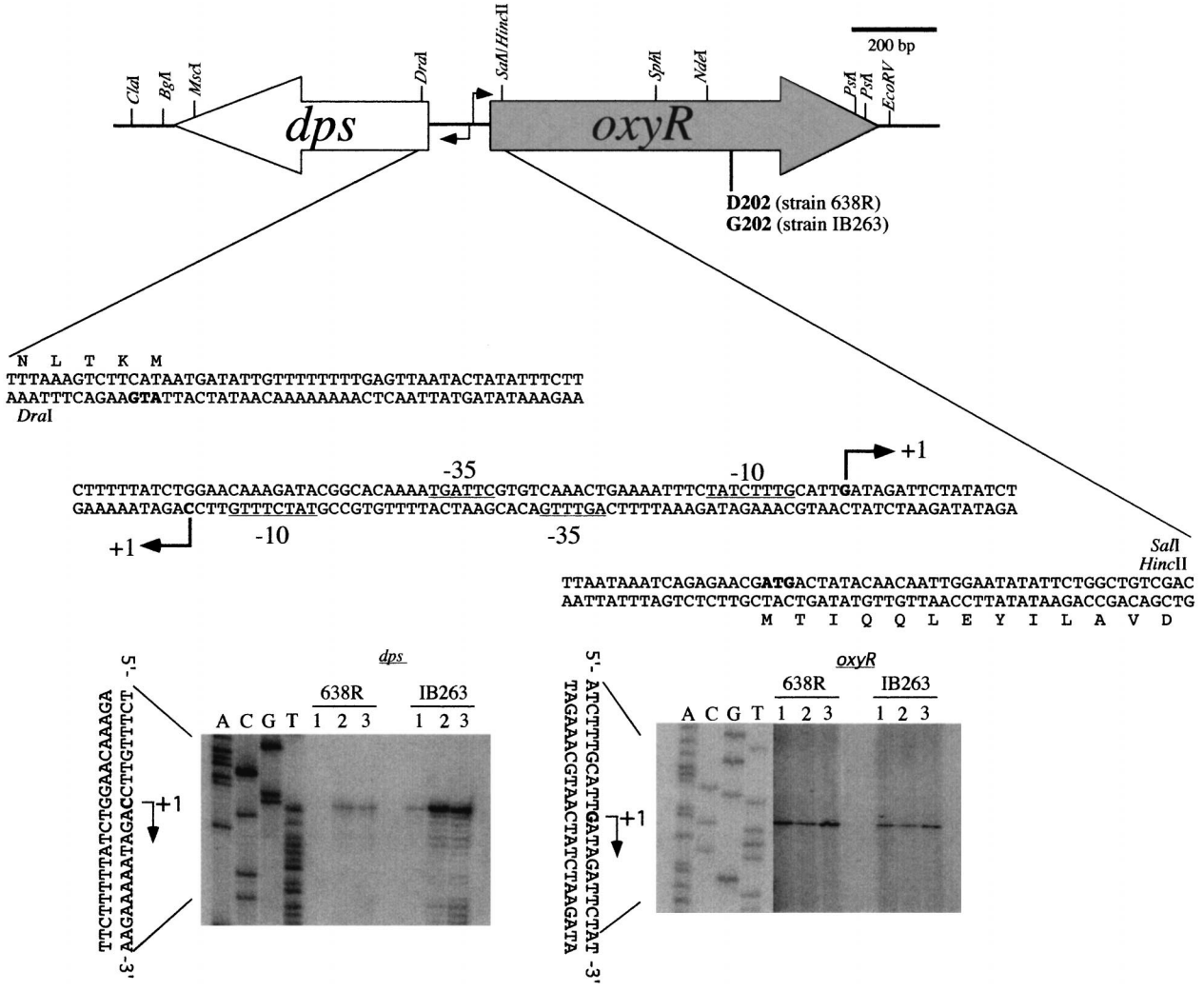

FIG. 2. Diagram of *oxyR* and *dps* genetic organization and structure of the promoter regions. The open and grey long arrows indicate the open reading frames and their respective direction of transcription. The *dps-oxyR* intergenic nucleotide sequence region and the first 5 codons of *dps* and 13 codons of *oxyR* also are shown. A partial restriction endonuclease map of the sequenced genes is indicated. The dark arrowheads indicate the transcription initiation nucleotide for *dps* and *oxyR* mRNAs. Based on a *B. fragilis* consensus (D. P. Bayley and C. J. Smith, unpublished data), the predicted −10 and −35 promoter region for each gene is underlined. The bottom panels show the primer extension autoradiographs used to determine the transcription start sites for *dps* and *oxyR* mRNAs. To the left of each panel is a DNA sequencing ladder generated with the same primer used for primer extension reactions. The following treatments were used as described in the Materials and Methods: anaerobic growth (lane 1), hydrogen peroxide treatment (lane 2), and oxygen exposure (lane 3).

OxyR(Con) is responsible for the constitutive regulation of the peroxide response in IB263. Figure 4D also shows that genetic complementation of the Δ*oxyR* strains with both *oxyR* and *oxyR*(Con) had no effect on the expression levels of *oxyR* compared to those in the parent strains (Fig. 5D). Taken together with data presented in Fig. 3C and 4D, these results demonstrate that *B. fragilis* OxyR is not involved in its own regulation.

Surprisingly, when Δ*oxyR* mutants were complemented with the wild-type *oxyR* gene, there was no induction of catalase activity by treatment with hydrogen peroxide or oxygen exposure compared to anaerobic cultures (Fig. 5C). In contrast, complementation of *B. fragilis* 638R Δ*oxyR* and IB263 Δ*oxyR* strains with *oxyR*(Con) (Fig. 5C) resulted in the constitutive expression of catalase activity, although there was no further induction by H_2O_2 treatment as seen with IB263 (Fig. 4C). The catalase activity was also abolished in the *ahpC*, *dps*, and *oxyR* β-xylosidase fusion strains when complemented with pFD772 following oxidative stress (data not shown). Moreover, constitutive expression of catalase activity was detected in all the β-xylosidase fusion strains complemented with pFD770 (data not shown). These finding indicated that multicopy *oxyR* was having an unexpected posttranscriptional effect on catalase. To test this hypothesis, pFD772 was mobilized into the 638R *katB′*::*xylB* strain (IB272), which carries the *katB* fusion integrated into the *bglA* gene and has a single copy of chromosomal *oxyR*. Then the β-xylosidase and catalase activities in the crude extracts of the new construct 638R *katB′*::*xylB* pFD772 were determined following oxidative stress (Fig. 6). The results show that induction of catalase activity was abolished in the strain carrying multicopy *oxyR* (Fig. 6A) while *katB* transcription was normally regulated as determined by *katB′*::*xylB* fusions (Fig. 6B). This suggests that multiple copies of *oxyR* in *B. fragilis* have a posttranscriptional effect on KatB when exposed to oxidative stress.

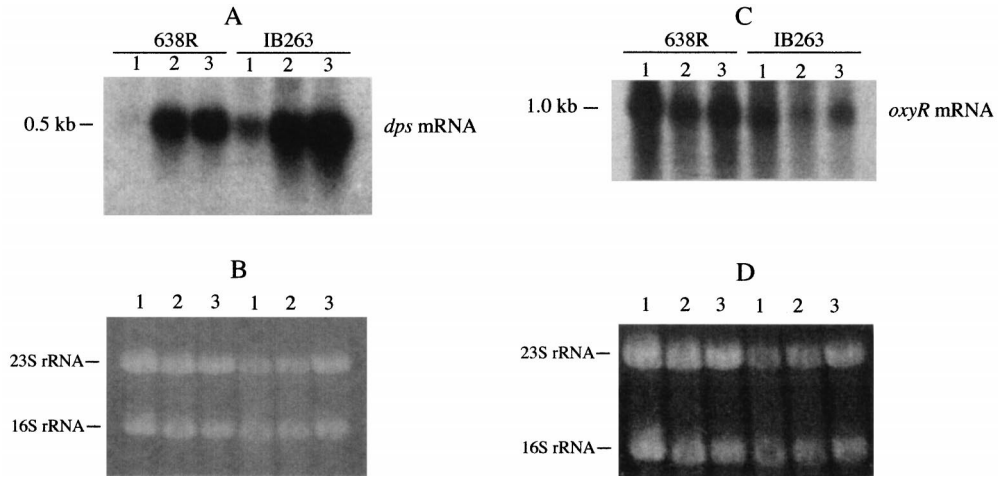

FIG. 3. (A and C) Autoradiographs of Northern hybridization membranes of total RNA from mid-log-phase *B. fragilis* 638R and IB263 following exposure to different oxidative stress conditions. The probe was a *dps* (A) or *oxyR* (C) internal gene fragment. Lanes: 1, anaerobic growth; 2, cultures treated with hydrogen peroxide; 3, cultures exposed to oxygen. The approximate sizes of the transcripts are indicated. (B and D) Respective ethidium bromide-stained agarose gels loaded with approximately 30 μg total RNA in each lane. The 23S and 16S rRNAs are also indicated.

DISCUSSION

Previous reports have shown that an oxidative stress response in the obligate anaerobe *B. fragilis* is inducible following treatment with hydrogen peroxide or oxygen exposure (22, 24, 30). In this study we show that the redox-sensitive transcriptional activator OxyR, a member of the LysR-type family of bacterial transcriptional activators (9, 36), is responsible for the control of the peroxide response regulon in this anaerobic microorganism. This is the first description of a functional oxidative stress response regulator in obligate anaerobic bacteria and in one so greatly diverged from the main line of eubacterial descent (41). We also show that deletion of *oxyR* resulted in loss of the peroxide-inducible response in both the parent strain and a constitutive peroxide-resistant mutant. Moreover, complementation of Δ*oxyR* with the *oxyR* gene restored the positive transcriptional activation of the peroxide response genes investigated and a point mutation in *oxyR* was linked to constitutive expression of these genes. Characteristic of the LysR-type family of transcriptional activators where the regulator is divergently transcribed from a gene it activates (29), *oxyR* was divergently transcribed from *dps*, a nonspecific DNA binding protein.

Strong evidence for control of the peroxide regulon by OxyR in *B. fragilis* is provided by the finding that the constitutive peroxide resistance phenotype of IB263 (26) is due to a mutated *oxyR* gene (GAT to GGT) at codon 202. It is likely that the D202G amino acid substitution near the redox-active C199 residue in IB263 resulted in a conformational change leading to a permanently activated form of OxyR, which is responsible for the constitutive expression of KatB, AhpC, and Dps. Other studies performed with *E. coli* have found that the A233V mutation is responsible for the constitutive *oxyR2* phenotype (9, 18). In another study, randomly mutagenized *oxyR* genes mapped to amino acid substitutions at the OxyR C-terminal domain conferred a permanent "locked" oxidized form of the protein. These mutations constitutively induced transcription under both reduced and oxidized conditions due to permanently induced cooperative binding of RNA polymerase (19, 38). It is interesting that mutation in the *B. fragilis oxyR* gene involved a C · G-to-T · A modification as occurred in all of the constitutive *E. coli oxyR* mutants investigated (18). This type of transition base substitution mutation is typical following oxidative DNA damage (17, 44), which probably occurred during the selection of IB263 for its increased hydrogen peroxide resistance.

As mentioned above, *B. fragilis* OxyR positively regulates the expression of the antioxidants KatB, AhpCF, and Dps as components of a set of approximately 28 oxidative stress proteins induced by hydrogen peroxide or oxygen exposure. These findings are similar to the peroxide response present in *E. coli* and *Senterica* serovar Typhimurium, where hydrogen peroxide induces the expression of a set of approximately 30 proteins (8, 10). Among these proteins, OxyR positively activates the transcription of nine antioxidant proteins including KatG, AhpCF, Dps, and GorA and a small regulatory RNA encoded by *oxyS* (2, 3, 8). Consistent with this role, preliminary experiments showed that the *B. fragilis* peroxide response protected primarily against peroxides, since there was only a small effect on the aerotolerance of Δ*oxyR* mutants (data not shown). Likewise, the *oxyR*(Con) mutant showed a much greater increase in its resistance to hydrogen peroxide killing than in its resistance to oxygen killing (26). It is interesting that although *B. fragilis* is an obligate anaerobic bacterium which cannot shift to an aerobic metabolism, it possesses a highly regulated peroxide response similar to the peroxide response reported to occur in aerobic and facultative bacteria.

In contrast to *E. coli* OxyR, which represses its own expression whether it is in the reduced or oxidized form (9, 37), we have found in this study that deletion of *oxyR* did not significantly alter the level of *oxyR* expression. Either *B. fragilis oxyR* is constitutively expressed or the autoregulatory mechanism does not allow sufficient alteration in *oxyR* expression levels to be detected by our *oxyR′*::*xylB* fusions. However, we think the evidence suggests that OxyR does not repress its own expression and is constitutively expressed. This is based on the facts that (i) basal levels of *oxyR* expression were not altered in the *oxyR* deletion mutants IB298 and IB299, (ii) *oxyR* expression was not altered following oxidative stress compared to anaerobic culture controls, and (iii) *oxyR* expression was not altered by complementation of Δ*oxyR* mutation with *oxyR* and *oxyR*-(Con) genes.

The *P. gingivalis* genomic database revealed that this phylo-

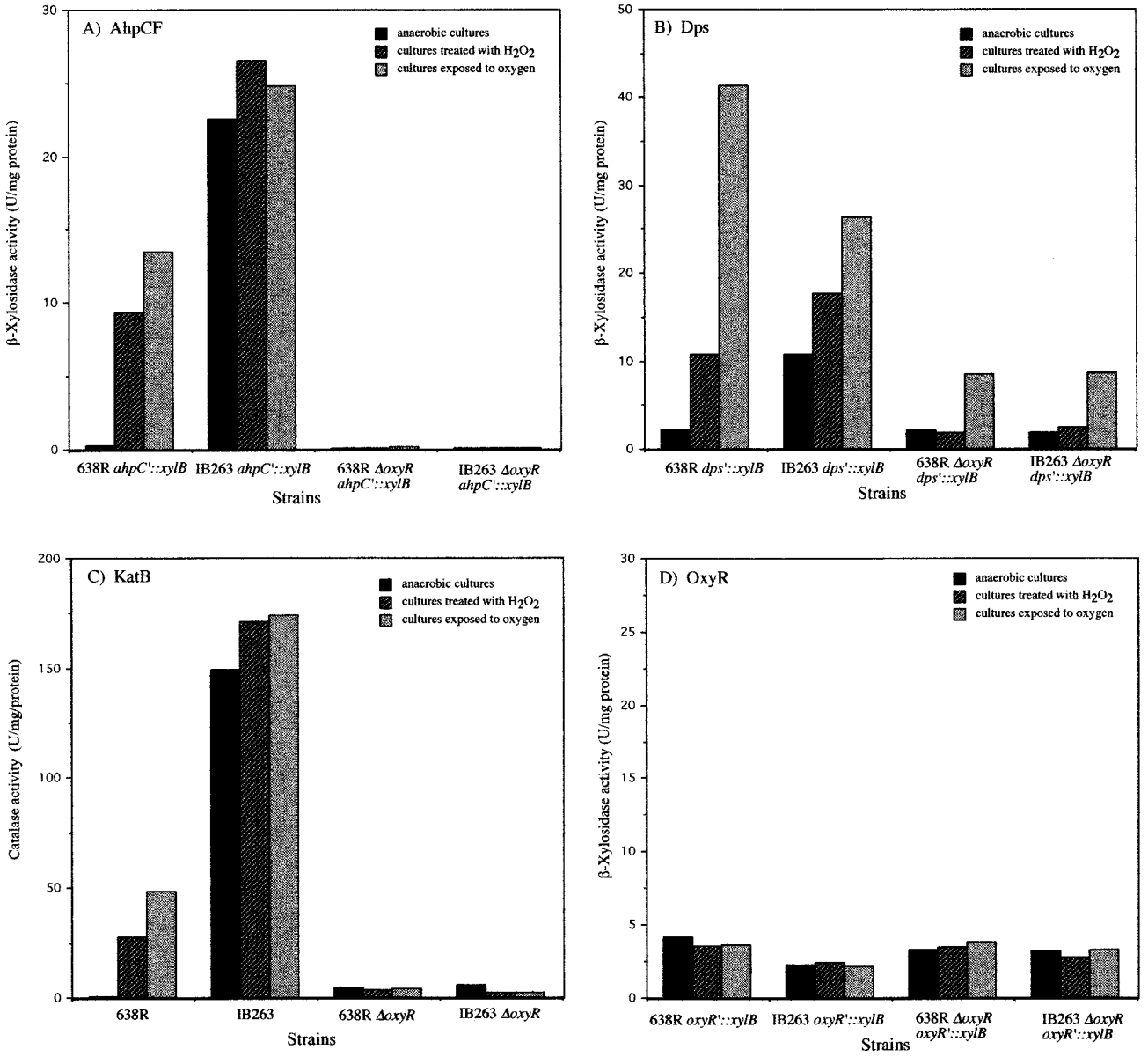

FIG. 4. Expression of peroxide-inducible genes in *oxyR* mutants. Determination of β-xylosidase (A, B, and D) and catalase (C) activities in crude extracts of mid-log-phase cells of *B. fragilis* parent and Δ*oxyR* mutant strains is shown. Bacteria were grown in BHIS and exposed to different oxidative stress conditions as indicated.

genetically related anaerobe also contained *oxyR* and *dps* and that these genes were closely related to the *B. fragilis* homologues. However, the genes are differently organized, with the *B. fragilis dps* and *oxyR* being divergently transcribed while the *P. gingivalis oxyR* is found in a head-to-tail arrangement with genes encoding an exodeoxyribonuclease and a single strand DNA binding protein (sequence data were obtained from the Institute for Genomic Research website at http://www.tigr.org). It is common for OxyR-regulated genes to be located adjacent to *oxyR*, but this is not always observed (9, 12, 15, 21, 37).

The role of OxyR in the control of the oxidative stress is well established, but there is some evidence that OxyR may be involved in other regulatory pathways which are apparently not involved directly in either scavenging oxygen radicals or repairing oxidative damage (5, 16, 40). In this regard, our finding that strains overproducing OxyR have repressed levels of catalase activity but not repressed transcription may suggest another role of OxyR in *B. fragilis*. That is, OxyR may be involved with iron or heme uptake, leading to the inactive catalase. Recent studies have shown that the *H. influenzae oxyR* mutant was unable to utilize protoporphyrin IX and had a reduced ability to incorporate heme (20). In *E. coli*, OxyR and SoxRS activate the expression of Fur, the global regulator of ferric iron uptake, suggesting that iron metabolism is coordinately regulated with the oxidative stress defenses (46). In this regard, the *B. fragilis* ferritin (*ftn*) gene was cloned and sequenced, and *ftn* expression was found to be up regulated in the parent strain and down regulated in a Δ*oxyR* mutant following oxidative stress (E. R. Rocha and C. J. Smith, unpublished results). This

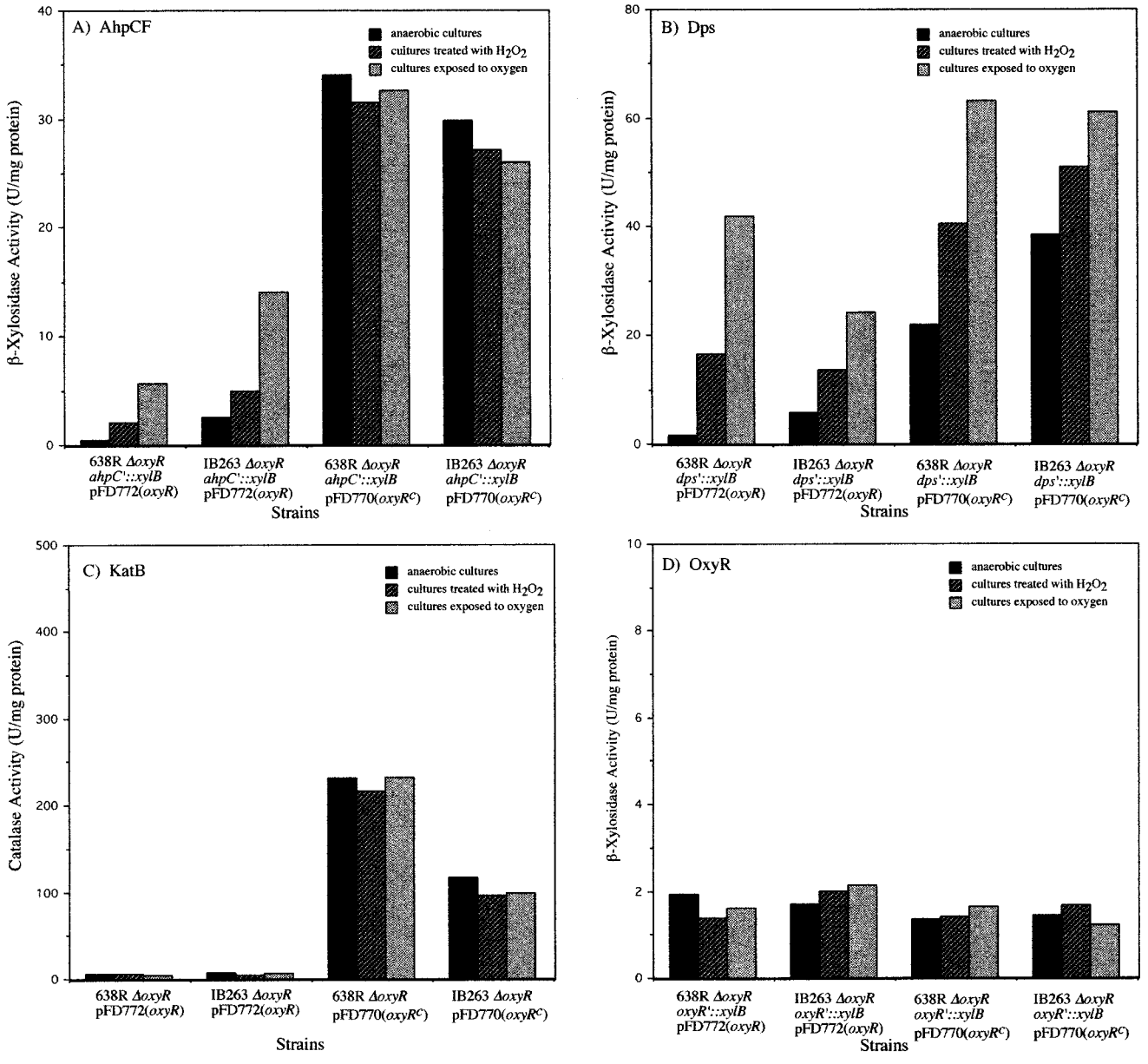

FIG. 5. Complementation of *oxyR* mutations. Determination of β-xylosidase (A, B, and D) and catalase (C) activities in crude extracts of mid-log-phase cells of *B. fragilis* Δ*oxyR* mutant strains completed with constitutive OxyR(Con), pFD770[*oxyR*(Con)], or wild-type OxyR, pFd772(*oxyR*) is shown. *oxyR*^{c} is equivalent to *oxyR*(Con). Bacteria were grown in BHIS and exposed to different oxidative stress conditions as indicated.

reinforces the idea that OxyR is involved in the mobilization of intra-cellular iron.

Although *dps* was divergently transcribed from *oxyR* and was controlled in large part by OxyR, we found that its regulation was more complex than expected, since it was up regulated by an oxygen-dependent, OxyR-independent mechanism in mid-log-phase cells (Fig. 4B) and by a different stationary-phase mechanism as determined by incorporation of radiolabeled methionine after 24 h of anaerobic growth (data not shown). This suggests that *B. fragilis dps* expression is under a multi-regulatory network that is able to activate the expression of this protein under different growth conditions. This seems to be a common characteristic in the regulation of *dps* in different organisms. In *E. coli*, *dps* expression is under the control of OxyR following the oxidative stress response and under the control of σ^S and integration host factor in the stationary phase (2). Dps also may be a link between oxidative stress and iron metabolism, as shown for *B. subtilis* (7). Thus, the presence of Dps in *B. fragilis* may be part of an important strategy to protect DNA under different environmental stress conditions.

It is worthwhile to note that OxyR and Dps from *B. fragilis* and *P. gingivalis* were clustered in the phylogenetic parsimony analysis in a branch separated from aerobic organisms, which suggests that many of the genes involved in the oxidative stress response were present in this anaerobic bacterium prior to its earlier diversion from other eubacteria (41). These two opportunistic human pathogenic anaerobic bacteria are well adapted to the strictly anaerobic environments of the human lower

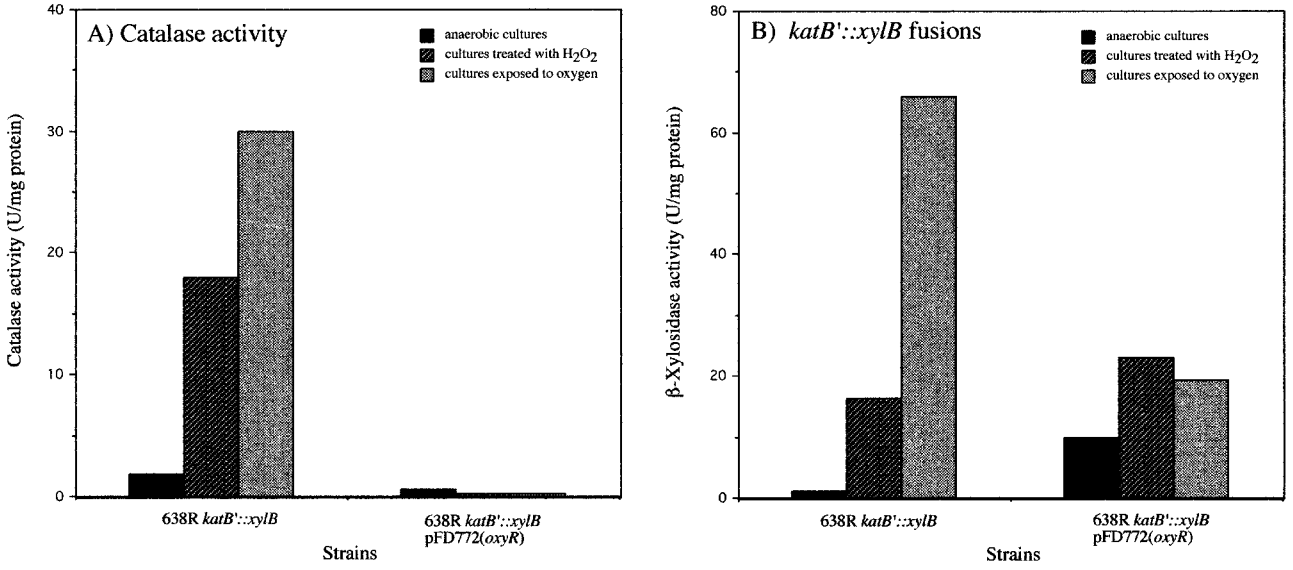

FIG. 6. Comparison of KatB enzyme activity to the expression of *katB* transcriptional fusion in OxyR-overproducing strains. Determination of catalase (A) and β-xylosidase (B) activities in crude extracts of mid-log-phase cells of *B. fragilis* 638R *katB'::xylB* transformed with pFd772(*oxyR*) is shown. Bacteria were grown to mid-log phase in BHIS and exposed to different oxidative stress conditions as indicated.

intestinal tract and gingival crevice, respectively; therefore, one must question the role of a complex oxidative stress response in these organisms. Perhaps it is a transitional mechanism used during the process of leaving their natural anaerobic environment to infect and colonize more oxygenated tissues as well as providing resistance to the oxidative burst of human phagocytes until appropriate anaerobic conditions are established at the site of infection.

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