Journal of Bacteriology, Nov. 1998, p. 5906–5912 Vol. 180, No. 22
0021-9193/98/$04.00+0
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Characterization of a Peroxide-Resistant Mutant of the Anaerobic Bacterium *Bacteroides fragilis*

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Received 14 May 1998/Accepted 3 September 1998

A *Bacteroides fragilis* mutant resistant to hydrogen peroxide and alkyl peroxide was isolated by enrichment in increasing concentrations of hydrogen peroxide. The mutant strain was constitutively resistant to 100 mM H_2O_2 and 5 mM cumene hydroperoxide (15-min exposure). In contrast, the parent strain was protected against <10 mM H_2O_2 when the peroxide response was induced with a sublethal concentration of H_2O_2, and no protection was observed in untreated cells. In addition, catalase activity in the mutant strain was not repressed in anaerobic cultures as reported previously for the parent strain. Comparison of the protein profile of crude extracts of the *B. fragilis* strains revealed that at least three oxidative stress-induced proteins in the parent strain were constitutively expressed in the mutant as detected by nondenaturing polyacrylamide gel electrophoresis. N-terminal amino acid sequence of these overexpressed proteins confirmed the presence of a deregulated catalase (KatB), an alkyl hydroperoxidase reductase subunit C (AhpC), and a Dps/PexB homologue. Northern blot analysis and *katB*::*cat* transcriptional fusion studies revealed that in the mutant, *katB* was deregulated compared to the parent and that *katB* was controlled by a *trans*-acting regulatory mechanism. Moreover, constitutive expression of KatB and of the AhpC and Dps homologues in the H_2O_2-resistant mutant suggests that these proteins may share a common oxidative stress transcriptional regulator and may be involved in *B. fragilis* peroxide resistance.

Microorganisms have developed highly efficient mechanisms that allow them to adapt rapidly and survive a variety of physical and chemical stress conditions such as oxygen availability, redox potential, temperature, pH, and osmolarity (33). One of these adaptations is the utilization of molecular oxygen as a final electron acceptor when facultative bacteria are shifted from anaerobic to aerobic conditions (45). Consequently, during aerobic growth, generation of the reactive oxygen species (ROS) superoxide anion (O_2^-) and of hydrogen peroxide (H_2O_2) is unavoidable, and the highly reactive oxidant hydroxyl radical ($OH^·$) also may be formed through the Fenton reaction of H_2O_2 with free transition metals such as ferrous iron (14). ROS are potent cellular oxidizing agents that damage proteins, membrane lipids, and DNA (14, 19, 40). To minimize this damage, microorganisms eliminate the harmful effect of oxygen by-products by the synthesis of ROS-scavenging enzymes such as superoxide dismutase, catalases, and peroxidases, oxidative damage-repairing enzymes, and other proteins with unknown functions (14, 40).

One important aspect of this ROS response is that treatment of facultative and aerobic bacteria with sublethal concentrations of H_2O_2 induces a protection of the cells against levels of H_2O_2 that would be otherwise lethal (4, 9, 10, 25). This peroxide response in *Salmonella typhimurium* and *Escherichia coli* results in the synthesis of at least 30 proteins, of which 9 are under OxyR regulatory control. These include catalase (KatG), alkyl hydroperoxide reductase (AhpCF), glutathione reductase (GorA), and a nonspecific DNA-binding protein (Dps/PexB) involved in protection of DNA against oxidative damage (1, 14, 20). Similarly, in response to both oxidative stress and stationary phase, *Bacillus subtilis* induces the synthesis of KatA, AhpCF, Dps and MrgA, an oxidative stress and metalloregulated Dps homologue (3, 8, 17).

Generally there is a paucity of information about the adaptive mechanisms that confer aerotolerance and survival of anaerobic bacteria in the presence of either oxygen or ROS. However, induction of an oxidative stress response has been shown to occur in the aerotolerant opportunistic human pathogen *Bacteroides fragilis* (29, 35, 37). *B. fragilis* is among the most aerotolerant of anaerobic bacteria and is able to resist the presence of molecular oxygen for up to 2 to 3 days without a significant loss of viability (32, 42). This aerotolerance is dependent on the ability to synthesize new proteins immediately following a shift to aerobic conditions or treatment with sublethal concentrations of H_2O_2 (29, 35, 37). At least 28 newly synthesized proteins are induced upon oxygen exposure or addition of exogenous H_2O_2 to mid-log-phase anaerobic cultures. Among these oxidative stress induced proteins are the ROS-scavenging enzymes catalase KatB (29) and superoxide dismutase (16), but the mechanism(s) regulating the synthesis of these proteins is not understood. Recently, we have shown that expression of the *B. fragilis katB* gene is transcriptionally regulated by oxidative stress and by carbon and energy limitation in the absence of oxygen (30). Investigations of the regulatory mechanisms as well as the roles that these proteins play in the aerotolerance of *B. fragilis* have led us to the isolation of a constitutive H_2O_2-resistant mutant. In this study, we present physiological and genetics analysis of this mutant which contribute to our understanding of the gene(s) involved and the regulation of inducible protection against peroxides in aerotolerant anaerobic bacteria.

MATERIALS AND METHODS

Strains and growth conditions. *B. fragilis* 638R (27) was grown anaerobically in brain heart infusion broth supplemented with hemin, cysteine, and $NaHCO_3$ (BHIS) for routine cultures (38). Cultures were also grown in chemically defined medium (44) for some enzyme analysis in crude extracts.

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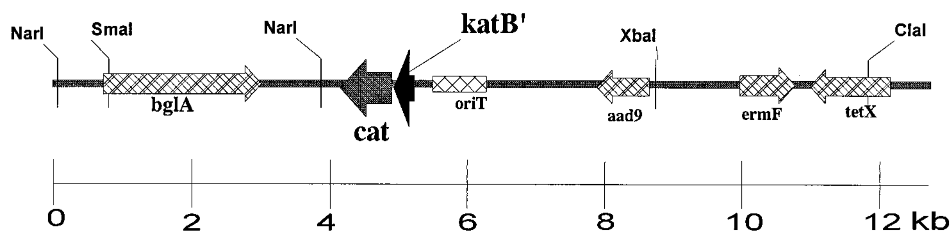

FIG. 1. Schematic diagram of the suicide shuttle vector pFD688 containing the transcriptional fusion *katB'*::*cat* and the *bglA* locus for targeted insertion into the chromosome of *B. fragilis*. Construction of the strains 638R (*katB*$^+$ *katB'*::*cat*) and IB263 (*katB*$^+$ *katB'*::*cat*) by using pFD688 is described in Materials and Methods.

Killing assays. Induction of the peroxide stress response with a sublethal concentration of H_2O_2 was carried out as follows. Mid-log-phase cells grown in BHIS to an A_{550} of 0.3 (approximately 2×10^8 to 4×10^8 cells/ml) were pretreated with 150 μM 2,2′-bipyridyl and 50 μM H_2O_2 for 15 min followed by a second addition of 50 μM H_2O_2 for 15 min. Treatment with bipyridyl was shown to be necessary for accurate viable counts (29). Then the cultures were split in 10-ml aliquots and challenged with H_2O_2 ranging from 0 to 100 mM for 15 min. For studies with cumene hydroperoxide (CHP), the cultures were pretreated as described above, and then CHP (in dimethyl sulfoxide [DMSO]) was added to final concentration of 0 to 5 mM. To determine viable counts, treated cultures were diluted in brain heart infusion broth containing 150 μM 2,2′-bipyridyl and 10 μg of bovine liver catalase per ml, plated on BHIS agar, and incubated for 3 to 5 days at 37°C. All procedures were performed within an anaerobic chamber. Exposure of anaerobic cultures to aerobic conditions was carried out as previously described (29).

Mutant selection. The procedure for isolation of the H_2O_2-resistant mutant described in this study was a modification of the continuous enrichment/selection method described by Hartford and Dowds (17). Briefly, *B. fragilis* 638R was grown in 10 ml of BHIS to approximately 2×10^8 to 4×10^8 cells/ml, treated with 10 mM H_2O_2 for 15 min, and then plated on BHIS agar for 24 to 48 h. One of the surviving clones was grown overnight in BHIS containing 10 mM H_2O_2. The resulting culture then was successively passaged overnight in BHIS containing 20, 30, 40, and then 50 mM H_2O_2. The *B. fragilis* culture resistant to 50 mM H_2O_2 was isolated on BHIS agar, and a single colony (designated IB263) was selected for further experiments.

Construction of a *katB*::*cat* transcriptional fusion. A *katB*::*cat* transcriptional fusion was constructed and integrated into the *B. fragilis* chromosome in order to study *trans*-acting regulation of the *katB* promoter. Briefly, a 0.8-kb blunt-ended *Taq*I DNA fragment containing the ribosome-binding site and coding region of the Tn*9* chloramphenicol acetyltransferase (*cat*) gene was ligated into the internal *Eco*RV and *Msc*I sites of *katB* in pFD567 (28). Restriction digestion of the new construct pFD605(*katB'*::*cat*) showed that the *cat* gene was in the same orientation as *katB*. Subsequently, a 2.5-kb *Sma*I/*Nar*I fragment from pFD605 (*katB'*::*cat*) was cloned into the unique *Sma*I/*Nar*I sites of the suicide vector pFD516 (39). Then a 3.8-kb *Nar*I fragment from pFD620 carrying *B. fragilis* β-glucosidase *bglA* gene (Genbank accession no. AF006658) was cloned in the unique *Nar*I site of the construct to provide a target for single-crossover-targeted insertion in the *B. fragilis* chromosome. The *bglA* gene is not essential for growth in BHIS, and there is at least one additional β-glucosidase gene in *B. fragilis* (31). The new construct, pFD688(*katB'*::*cat*) (Fig. 1), was mobilized from *Escherichia coli* DH5α by triparental mating into *B. fragilis* 638R and IB263, using standard filter mating protocols (36). Southern blotting hybridization analysis revealed that pFD688 was integrated into the *B. fragilis bglA* locus, creating strains 638R (*katB*$^+$ *katB'*::*cat*) and IB263 (*katB*$^+$ *katB'*::*cat*). Total RNA extraction and Northern blot analysis of *katB* mRNA were carried out exactly as previously described (30). The densitometry analysis of the autoradiograph was normalized to the relative intensity of total 23S and 16S rRNAs detected on the ethidium bromide-stained agarose gel.

Catalase and CAT enzyme assays. Catalase activity was measured spectrophotometrically as previously described (28). One unit of catalase is the amount of enzyme which decomposes 1 μmol of H_2O_2 per min at 25°C. The spectrophotometric assay for CAT was performed in crude extracts essentially as described by Brosius and Lupski (7).

Partial protein purification and PAGE. Partial purification of oxidative stress proteins was obtained by electroelution of the bacterial crude extract on a polyacrylamide gel at 40-mA constant current, using a Prep-Cell model 491 (Bio-Rad Laboratories, Inc., Melville, N.Y.). Fractions containing the proteins of interest were pooled and concentrated. Preparative and analytical nondenaturing polyacrylamide gel electrophoresis (PAGE) and denaturing sodium dodecyl sulfate (SDS)-PAGE were performed as described by Laemmli (21). Following electrophoresis, proteins were detected by staining the gel with either Coomassie blue R250 or Ponceau S. Protein concentration was determined by the Bradford method (6), using lysozyme as a standard.

N-terminal amino acid sequence and database comparison. The proteins resolved by SDS-PAGE were blotted to a polyvinylidene difluoride membrane in 10 mM CAPS (3-[cyclohexylamino-1-propanesulfonic acid])–10% methanol, pH 11.0 (23). The blotted proteins were subjected to fully automated solid-phase Edman degradation to determine the N-terminal amino acid sequence. The N-terminal sequencing was performed by D. Klapper, University North Carolina, Chapel Hill. Computer analyses of N-terminal amino acid sequences were performed with the University of Wisconsin Genetics Computer Group DNA sequence analysis software (11).

RESULTS

Isolation and characterization of a hydrogen peroxide-resistant mutant. A *B. fragilis* hydrogen peroxide-resistant strain, IB263, was isolated following enrichment of the 638R cultures in increasing concentrations of H_2O_2. After several passages in BHIS in the absence of H_2O_2, cell viable counts were performed to determine whether resistance to H_2O_2 was constitutively expressed in IB263. The mutant strain IB263 was no longer killed by passage in media containing as much as 50 mM H_2O_2, suggesting that there was constitutive resistance to H_2O_2 due to a stable mutation(s) rather than a reversible and temporary physiological adaptation. This possibility was supported by the results in Fig. 2A, where it is clearly shown that IB263 was highly resistant up to 100 mM H_2O_2 with or without H_2O_2 induction. In contrast, the parent strain lost about 2 orders of magnitude in viability in the presence of 5 mM H_2O_2 when the peroxide response was induced, and there was essentially no protection in untreated cells.

The results in Fig. 2B showed that IB263 also was constitutively resistant to the organic peroxide CHP up to 5 mM in the presence of bipyridyl. In the parent strain, resistance to higher concentrations of CHP required induction by pretreatment with H_2O_2. Pretreatment of the parent cultures with 50 μM CHP induced a similar response, suggesting that either hydrogen or alkyl peroxide is able to induce resistance to organic peroxide (data not shown). In contrast to the increased peroxide resistance, the mutant strain was not altered or slightly more sensitive to molecular oxygen compared to the parent strain (Fig. 3). Mid-log-phase cells of the peroxide-resistant strain exposed to oxygen for 48 h had about a 5-log decrease in the number of viable cells compared to that of the parent culture control, which took 72 h to decrease to approximately the same number of survival cells. Stationary-phase mutant cells shifted to aerobic conditions also were slightly more sensitive to oxygen than the parent, but overall stationary-phase cells were less sensitive to oxygen than log-phase cells. No apparent difference was noted for the anaerobic condition culture controls.

Deregulation of catalase activity and evidence for a *trans*-acting regulatory mechanism. Previous work had shown that induction of catalase was essential for full protection against exogenous H_2O_2 (29). Thus, to investigate whether constitutive resistance to H_2O_2 was correlated to an increase in catalase activity, both parent and mutant strains were tested by catalase assays (Fig. 4A). Catalase activity of approximately 5,100 U/mg of protein was found in crude extracts of the mutant strain

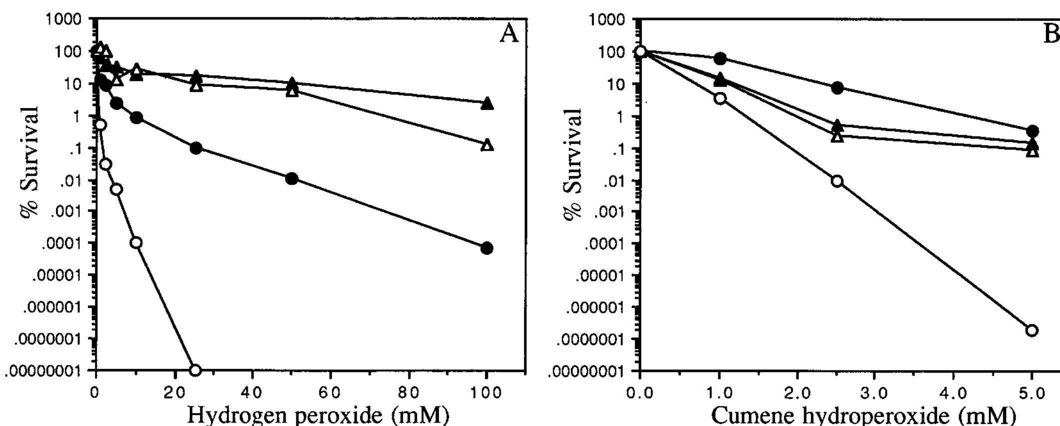

FIG. 2. Survival of mid-log-phase cells of *B. fragilis* 638R and IB263 following addition of hydrogen peroxide (A) and CHP (B) for 15 min. Data points represent *B. fragilis* 638R cultures pretreated (●) and not pretreated (○) with H_2O_2 and *B. fragilis* IB263 cultures pretreated (▲) and not pretreated (△) with H_2O_2.

grown in chemically defined medium, indicating that it was no longer repressed under anaerobic conditions. There was a >180-fold increase over the much lower activity (27 U/mg protein) found in the anaerobic cultures of the parent strain, suggesting that the mechanism(s) that controls catalase expression in the parent strain is no longer functional in the mutant. In addition, there was no further significant induction of catalase activity when anaerobic cultures of IB263 were exposed to oxygen or treated with hydrogen peroxide (6,700 and 5,600 U/mg of protein, respectively). In contrast there was approximately a 15-fold-higher activity induced in the parent strain following exposure to the same oxidative stress conditions (170 and 360 U/mg of protein, respectively). As the catalase regulation in the parent strain occurs at the transcriptional level (30), Northern blot hybridization analysis of the mutant was carried out. The results revealed that the level of *katB* mRNA present in strain IB263 under anaerobic conditions was approximately 20-fold higher than in the anaerobic culture of the parent strain (Fig. 5). *katB* mRNA obtained from the mutant exposed to oxygen or treated with hydrogen peroxide showed a slight increase in expression which was similar to the induction seen for the parent strain (between 30- and 35-fold increase). These results strongly suggest that the high levels of catalase in the mutant strain were due to the transcriptional deregulation of *katB* mRNA expression.

The regulation was further investigated by using a *katB*::*cat* transcriptional fusion integrated into the *bglA* locus of both the parent and the mutant strain. The CAT activities in crude extracts of mid-log-phase cells of strains 638R (*katB*$^+$ *katB*′::*cat*) and IB263 (*katB*$^+$ *katB*′::*cat*) are shown in Fig. 4B. There was an increase of approximately 200-fold in CAT activity in the mutant strain (219 U/mg of protein) compared to the anaerobic culture of the parent strain (0.8 U/mg of protein). No further induction by oxygen exposure and treatment with H_2O_2 (210 and 190 U/mg of protein, respectively) was observed in IB263; in contrast, CAT activity was induced by oxidative stress conditions in the parent strain. Comparison of data for the parent and mutant strains in Fig. 4 clearly shows that the mutant lost the wild-type regulation in both the *katB*$^+$ gene and the *katB*′::*cat* fusion. These results indicate that the deregulation of catalase expression is due to a mutation in a *trans*-acting regulatory element rather than a *cis*-acting mutation in the *katB* regulatory region.

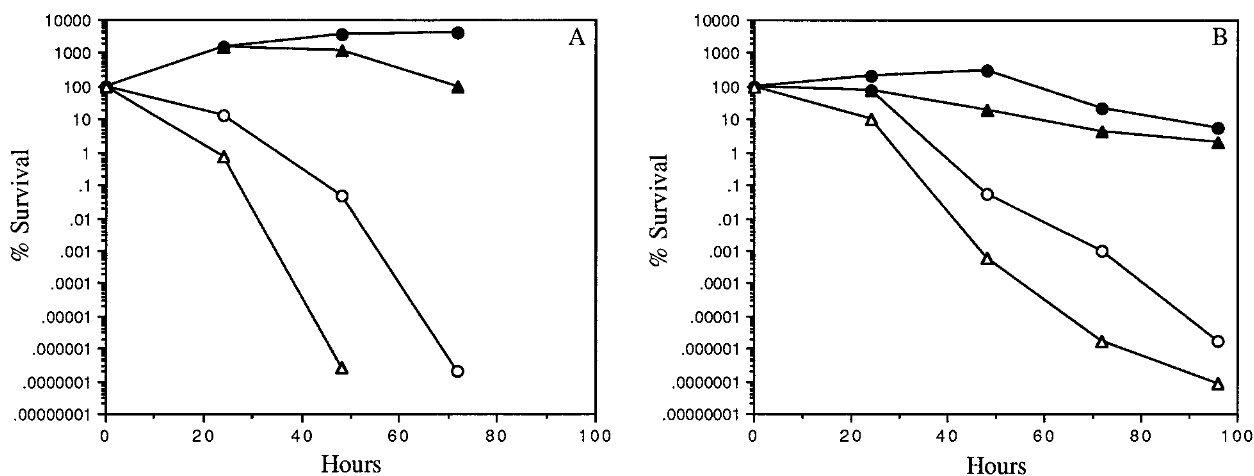

FIG. 3. Survival of anaerobic *B. fragilis* 638R (●) and IB263 (▲) mid-log-phase (A) and stationary-phase (B) cells shifted to aerobic conditions (○ and △, respectively). Cultures of mid-log-phase cells (A_{550} = 0.3) or early-stationary-phase cells (A_{550} = 1.1) were divided at time zero; one half was shaken at 250 rpm in air, and the other half was maintained anaerobically. Viable cell counts were determined at the times shown.

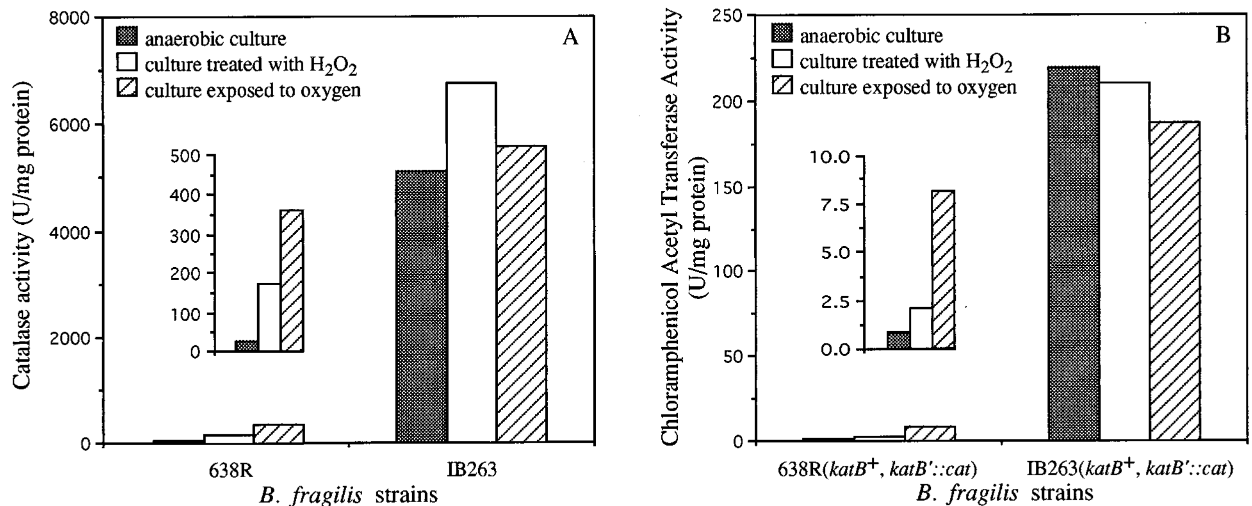

FIG. 4. Catalase (A) and CAT (B) activities in crude extracts of mid-log-phase cells of *B. fragilis* strains grown in chemically defined medium under different oxidative stress conditions. The insets show amplified scales of the *B. fragilis* 638R catalase and CAT activities.

Identification of overexpressed proteins in IB263. The data from the H_2O_2 and CHP survival studies suggested that the regulatory mutation in IB263 may affect the global oxidative stress response. Therefore, additional studies were performed to see if other oxidative stress proteins were constitutively expressed in the mutant strain. Nondenaturing PAGE of crude extracts from *B. fragilis* 638R and IB263 exposed to oxygen and hydrogen peroxide revealed several candidate oxidative stress proteins (Fig. 6). One overexpressed protein had an electrophoretic mobility identical to that of *B. fragilis* catalase KatB previously purified (28), which together with the results shown above confirms our findings showing that catalase is overexpressed in the mutant strain.

Two of the overexpressed proteins were characterized further following partial purification using preparative nondenaturing PAGE and SDS-PAGE. One protein had a single-subunit molecular weight of approximately 18,000 as determined by SDS-PAGE; the protein was electroblotted onto a polyvinylidene difluoride membrane, and the N-terminal amino acid sequence was determined. Alignment of the first 30 amino acid residues of the N terminus with amino acid sequences available from the GenBank database showed similarity to the Dps protein family of DNA-binding proteins (Fig. 7A). Comparison of the *B. fragilis* Dps-like protein to members of the Dps group of proteins revealed 27% identity (38% similarity) to *E. coli* Dps/PexB protein, 21% identity (38% similarity) to an *Haemophilus influenzae* Dps homologue, 30% identity (33% similarity) to *Synechococcus* strain PCC7942 nutrient stress-induced DNA-binding hemoprotein (DpsA), 23% identity (37% similarity) to an *Anabaena variabilis* low-temperature-induced protein, 25% identity (35% similarity) to *Helicobacter pylori* neutrophil-activating protein A (NapA; bacterioferritin), 20% identity (30% similarity) to *B. subtilis* MrgA, and 23% identity (30% similarity) to *Listeria innocua* nonheme iron-binding ferritin.

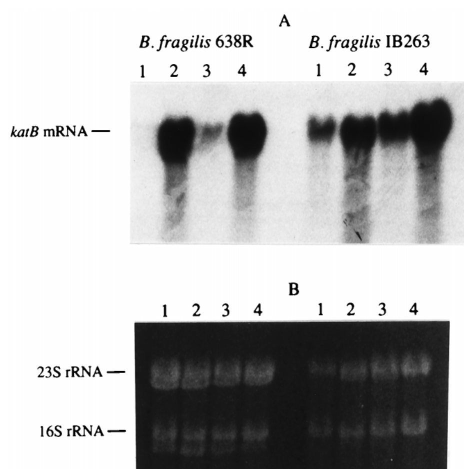

FIG. 5. Northern hybridization analysis of mid-log-phase *B. fragilis* 638R and IB263 cells under different oxidative stress conditions. (A) Autoradiograph of a Northern blot probed with the *katB* internal fragment. (B) Ethidium bromide-stained agarose gel loaded with 30 μg of total RNA in each lane. The 23S and 16S rRNAs are also indicated. Lanes: 1, anaerobic cultures; 2, cultures treated with 500 μM H_2O_2; 3, culture treated with 1 mM potassium ferricyanide; 4, cultures exposed to aeration for 1 h.

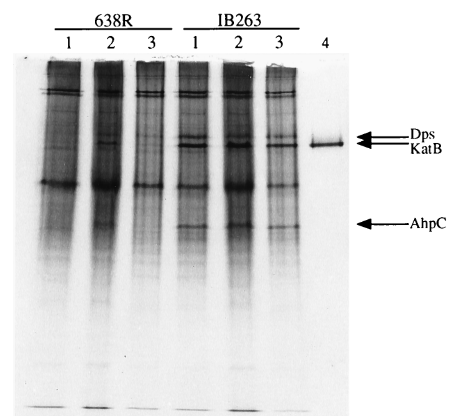

FIG. 6. Nondenaturing PAGE (7.5 to 20% gradient polyacrylamide gel) of mid-log-phase crude extracts of *B. fragilis* 638R and IB263. Lanes: 1, anaerobic cultures; 2, anaerobic cultures pretreated with H_2O_2; 3, anaerobic cultures exposed to oxygen for 1 h; 4, approximately 2.5 μg of purified *B. fragilis* catalase KatB (28). Lanes 1 to 3 were loaded with approximately 50 μg of total protein; the gel was stained with Coomassie blue. Arrows show the major protein bands that are overexpressed in the mutant strain.

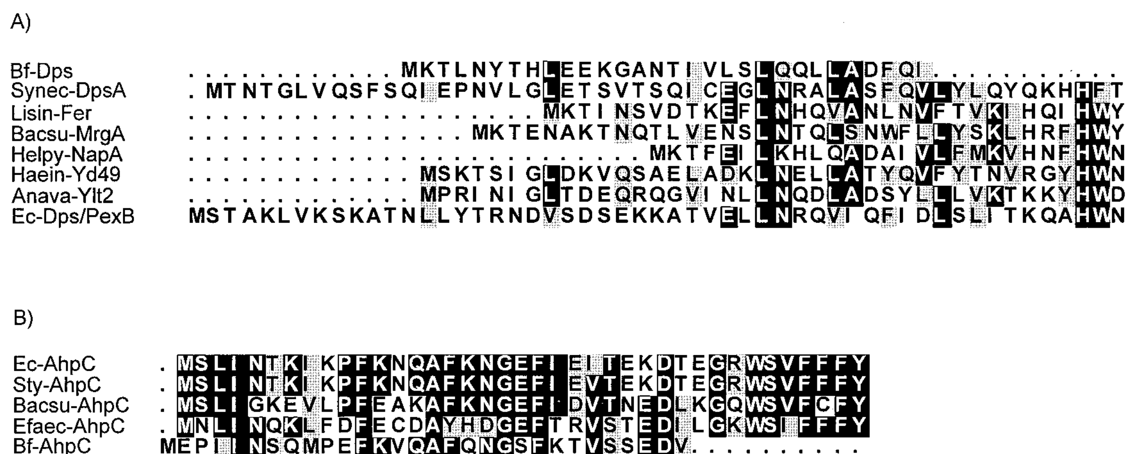

FIG. 7. (A) Alignment of the N-terminal amino acid sequence of a *B. fragilis* Dps homologue (Bf-Dps) with those of *Synechococcus* strain PCC7942 (Synec-DpsA) (26), nonheme iron-binding ferritin of *L. innocua* (Lisin-Fer) (5), *B. subtilis* MrgA (Bacsu-MrgA) (8), *H. pylori* NapA (Helpy-NapA) (13), *H. influenzae* (Haein-YD49; GenBank accession no. P45173), *A. variabilis* low-temperature-induced protein (Anava-YLT2; GenBank accession no. P29712), and *E. coli* Dps/PexB (Ec-Dps/PexB) (1). (B) Alignment of the N-terminal amino acid sequence of a *B. fragilis* AhpC homologue (Bf-AhpC) with those of *E. coli* AhpC (Ec-AhpC; GenBank accession no. D90701), *S. typhimurium* AhpC (Sty-AhpC; GenBank accession no. P19479), *B. subtilis* AhpC (Bacsu-AhpC) (17), and *E. faecalis* AhpC (Efaec-AhpC; GenBank accession no. AF016233). Consensus of at least 50% identical amino acid residues is denoted by black boxes; conserved amino acid substitutions are depicted by grey boxes.

Another constitutively expressed protein that was characterized had a single subunit with a molecular weight of approximately 22,000 (data not shown). Alignment of the first 30 N-terminal amino acids revealed strong homology to bacterial alkyl hydroperoxide reductase subunit C (AhpC) and to the thiol-specific antioxidant family of antioxidant proteins from prokaryotes and eukaryotes. Alignment of *B. fragilis* AhpC with other bacterial alkyl hydroperoxide reductase subunit C is shown in Fig. 7B. The N-terminal amino acid sequence of *B. fragilis* AhpC revealed 39% identity (46% similarity) to *E. coli* AhpC, 43% identity (46% similarity) to *S. typhimurium* AhpC, 34% identity (41% similarity) to *B. subtilis* AhpC, and 35% identity (52% similarity) to *Enterococcus faecalis* AhpC.

DISCUSSION

When mid-log-phase cells of *B. fragilis* are shifted from anaerobic to aerobic conditions, they are no longer able to maintain cell division but cell viability remains high even after oxygen exposure for up to 3 days. We are interested in understanding the physiological adaptations that occur during this period of reversible growth arrest under aerobic conditions. The initial studies suggested that the ROS-scavenging enzymes superoxide dismutase and catalase were important for aerotolerance in *B. fragilis* (24). More recently, Rocha et al. (29) have shown that response to oxidative stress in *B. fragilis* induces the synthesis of at least 28 proteins in the presence of oxygen or hydrogen peroxide. This finding is very interesting since this obligate anaerobe is not able to shift to an aerobic metabolism and maintain growth under aerobic conditions but does respond with the synthesis of a protective mechanism against the toxic effects of ROS. In this study, we describe the isolation and partial characterization of a *B. fragilis* H_2O_2- and organic peroxide-resistant mutant and identification of constitutively expressed proteins that may be potentially involved in protection against ROS in anaerobic bacteria.

The resistance to high levels of H_2O_2 in mutant strain IB263 was correlated with constitutive catalase activity, and the important role that KatB plays in protection against an exogenous source of H_2O_2 is consistent with this observation (29). In addition, evidence showing that *B. fragilis* produces homologues of AhpC and Dps, which are known to be involved in resistance to peroxides and oxidative DNA damage, was presented (1, 22, 41). Taken together, the constitutive resistance to hydrogen peroxide and organic peroxide and the overexpression of KatB, AhpC, and Dps in IB263 suggest that these activities are linked to a peroxide resistance response and may be under a common transcriptional regulator. Strong support for this idea was provided by the promoter fusion experiments. The findings in Fig. 4B clearly showed that the wild-type *katB* promoter was deregulated in IB263 but regulated normally in the parent, which suggests that a *trans*-acting regulatory mechanism controlling the peroxide response exists in *B. fragilis*. However, at this point it is not possible to rule out the formal possibility that the deregulation of KatB, AhpC, and Dps resulted from multiple mutations induced by the H_2O_2 enrichment technique. This matter will be clarified in further studies on complementation or isolation of the mutation.

The peroxide response in facultative and aerobic bacteria has been extensively studied and found to be complex and tightly regulated. In *E. coli* and *S. typhimurium*, the H_2O_2 redox sensor and transcriptional activator OxyR induces in mid-log-phase cells the synthesis of nine proteins, including KatG, AhpCF, GorA, and Dps (2, 9). A constitutive OxyR mutant confers overexpression of these proteins and resistance to hydrogen peroxide (9). More recently, Zheng et al. (47) have shown that two OxyR conserved cysteine residues specifically sense peroxides, forming disulfide bonds leading to intramolecular conformational changes and activation of OxyR. In *B. subtilis*, a hydrogen peroxide-resistant mutant overexpresses KatA, AhpC, and MrgA (a metalloregulated Dps homologue), possibly due to a mutation in a transcriptional repressor (17) that may contain a redox-active metal ion cofactor (8). Thus, there are a variety of mechanisms able to sense oxidative stress in cells, and it will be of interest to determine the mode of control in anaerobic organisms.

Dps protein has been found in several aerobic organisms (1, 5, 8, 13, 26, 46), and one of its major functions is to protect DNA against oxidative damage (22). However, Dps may participate in gene regulation in stationary-phase and in peroxide-

consuming reactions located at the chromosome (1, 26). Dps protein is related to the ferritin/bacterioferritin family of iron storage proteins, suggesting the possibility of divergence from a common ancestor (5, 26). Moreover, both AhpC and Dps in *S. typhimurium* (15, 43) and AhpC in *Mycobacterium tuberculosis* (12) were induced during interactions with macrophages, suggesting that these oxidative stress proteins may participate in survival to the macrophage oxidative burst killing mechanisms. These findings raise several questions as to how anaerobic bacteria have acquired genes involved in detoxification and protection against toxic ROS since they are adapted to live in the absence of molecular oxygen.

Despite the high resistance to hydrogen peroxide in *B. fragilis* IB263 under anaerobic conditions, there is no corresponding increase in resistance to oxygen exposure. In contrast, it seems that the mutant strain was slightly more sensitive to oxygen than the parent strain. Although the peroxide response may be effective in scavenging and detoxifying peroxides formed during oxygen exposure, it is not clear whether this deregulated peroxide response would eventually affect other protective mechanisms, such as a superoxide response, that might be involved in controlling aerotolerance in this strain. On the other hand, it is possible that other secondary mutations affecting the sensitivity to oxygen are present in IB263. However, evidence supporting the possibility that oxygen toxicity is somehow different from the toxicity exerted by the H_2O_2 is available from previous studies showing that the effect of H_2O_2 on the degradation and repair of DNA differs from the effect of oxygen in irradiated *B. fragilis* cells (34, 37). *B. fragilis* Bf-2 was more sensitive to DNA-damaging agents such as far-UV radiation, *N*-methyl-*N'*-nitrosoguanidine, ethyl methanesulfonate, acriflavine, and mitomycin C in the presence of oxygen than when treated with H_2O_2. Aerotolerance and resistance to ROS in anaerobic bacteria may be part of a more complex mechanism which still remains to be elucidated. In this regard, we have identified several classes of genes that are differentially induced by various oxidative stresses (unpublished), and this investigation will be the focus of a future report.

ACKNOWLEDGMENTS

This work was supported in part by grant 9513-ARG-0038 from the North Carolina Biotechnology Center and PHS grant AI-28884.

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