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# Oxidative Stress Response in an Anaerobe, *Bacteroides fragilis*: a Role for Catalase in Protection against Hydrogen Peroxide

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**Survival of *Bacteroides fragilis* in the presence of oxygen was dependent on the ability of bacteria to synthesize new proteins, as determined by the inhibition of protein synthesis after oxygen exposure. The *B. fragilis* protein profile was significantly altered after either a shift from anaerobic to aerobic conditions with or without paraquat or the addition of exogenous hydrogen peroxide. As determined by autoradiography after two-dimensional gel electrophoresis, approximately 28 newly synthesized proteins were detected in response to oxidative conditions. These proteins were found to have a broad range of pI values (from 5.1 to 7.2) and molecular weights (from 12,000 to 79,000). The hydrogen peroxide- and paraquat-inducible responses were similar but not identical to that induced by oxygen as seen by two-dimensional gel protein profile. Eleven of the oxidative response proteins were closely related, with pI values and molecular weights from 5.1 to 5.8 and from 17,000 to 23,000, respectively. As a first step to understanding the resistance to oxygen, a catalase-deficient mutant was constructed by allelic gene exchange. The *katB* mutant was found to be more sensitive to the lethal effects of hydrogen peroxide than was the parent strain when the ferrous iron chelator bipyridyl was added to culture media. This suggests that the presence of ferrous iron in anaerobic culture media exacerbates the toxicity of hydrogen peroxide and that the presence of a functional catalase is important for survival in the presence of hydrogen peroxide. Further, the treatment of cultures with a sublethal concentration of hydrogen peroxide was necessary to induce resistance to higher concentrations of hydrogen peroxide in the parent strain, suggesting that this was an inducible response. This was confirmed when the bacterial culture, treated with chloramphenicol before the cells were exposed to a sublethal concentration of peroxide, completely lost viability. In contrast, cell viability was greatly preserved when protein synthesis inhibition occurred after peroxide induction. Complementation of catalase activity in the mutant restored the ability of the mutant strain to survive in the presence of hydrogen peroxide, showing that the catalase (KatB) may play a role in oxidative stress resistance in aerotolerant anaerobic bacteria.**

In the presence of oxygen, aerobic and facultative bacteria utilize molecular oxygen as a terminal electron acceptor. As a consequence of one-electron reduction steps from oxygen to water, the reactive oxygen species superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) are formed (6, 24). Moreover, in the Fenton reaction, free hydrogen peroxide readily reacts with available transition metals such as ferrous iron to form a more powerful and highly reactive oxidant, the hydroxyl radical ($OH^\bullet$) (14, 35). Superoxide anion and hydrogen peroxide may also be generated by autoxidation of dehydrogenases, catechols, thiols, flavins, and oxidases and by UV radiation (4). Oxygen radicals are implicated in severe damage to membrane lipids, proteins, and DNA (4, 17, 41). In order to prevent this damage, microorganisms have developed efficient mechanisms to eliminate harmful oxygen by-products. Superoxide dismutase (SOD) eliminates toxic $O_2^-$ by dismutation to $H_2O_2$ and $O_2$ (6), and the accumulation of toxic $H_2O_2$ is prevented by the action of catalases and peroxidases (15).

On the other hand, under anaerobic conditions, the generation of reactive oxygen species (ROS) is not thought to be a problem because of the absence of oxygen. The lack of protective mechanisms against oxygen toxicity in anaerobic bacteria was seen as an explanation for their sensitivity to oxygen exposure (24). Many studies have shown that anaerobic bacteria are not uniformly sensitive to oxygen, and there is a broad range of oxygen tolerance from species that are extremely sensitive to those that are able to remain viable in the presence of oxygen for extended periods (5, 24). It is believed that for some anaerobic bacteria, as in aerobic organisms, the presence of SOD and catalase play a role in the detoxification of ROS (24).

Among anaerobic bacteria, the opportunistic pathogen *Bacteroides fragilis* is one of the most aerotolerant species; this aerotolerance may prove to be an important virulence factor. This is supported by the fact that clinical isolates of *B. fragilis* were more resistant to oxygen exposure than were fecal strains (34, 42). The former strains were able to survive in the presence of oxygen for 48 to 72 h, while the latter lost total viability after 4 h of oxygen exposure. These results suggest that a system directly related to oxygen tolerance or oxidative stress exists, and several lines of evidence from several laboratories are consistent with this idea. For example, *B. fragilis* cells treated with sublethal concentrations of $H_2O_2$ or exposed to oxygen were more resistant to challenge with lethal concentrations of peroxide than were untreated cells (38). This response includes both SOD and catalase, which have been found to be induced by oxygen in *B. fragilis* (9–12, 31, 33, 47). In addition, treatment with either far-UV radiation, oxygen, or $H_2O_2$ has been shown to induce the synthesis of three, six, and four proteins, respectively, as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *B. fragilis* cell extracts (36). Other systems, such as the production of a ferritin protein which may alleviate iron toxicity (32) and the

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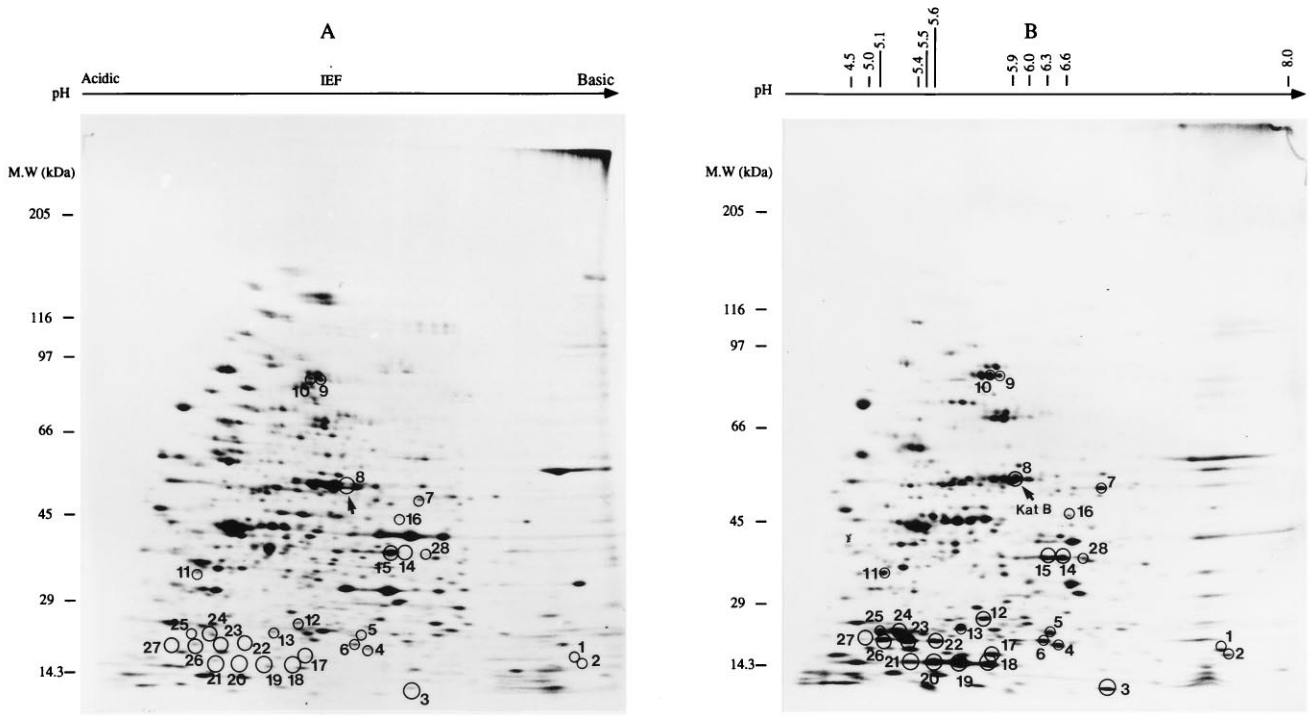


FIG. 1. Autoradiographs of 2-D electrophoresis of extracts from late-log-phase cells of *B. fragilis* 638R. (A) Anaerobic culture; (B) culture exposed to oxygen. The standard 2-D protein map of an oxygen-exposed culture of *B. fragilis* 638R is shown in panel B. The aerobic induced proteins are marked by open circles and are numbered as shown in Fig. 2. The KatB position is indicated by an arrow. M.W, molecular weight; IEF, isoelectric focusing.

synthesis of ppGppp in the presence of oxygen (7), may also be important.

Nevertheless, there is a paucity of information on the general physiological effects of a shift of *B. fragilis* cells from anaerobic to aerobic environments. It likely would be an oversimplification to assume that the presence of ROS-scavenging enzymes alone is responsible for oxygen tolerance in anaerobic bacteria. Thus, we initiated studies to understand how *B. fragilis* is protected from the toxic effects of oxygen exposure and $H_2O_2$. In this work, we describe the response of *B. fragilis* to oxygen and $H_2O_2$ exposure at the level of protein synthesis. We show that a specific oxidative stress response is induced by both oxygen and $H_2O_2$ and that there is considerable overlap in the responses. This response was protective, as determined by viable-cell counts of cultures exposed to oxygen or $H_2O_2$. Finally, the role of one of the induced proteins, catalase KatB, was examined. A *katB* mutant was found to be more sensitive to the lethal effects of exogenous $H_2O_2$, but there was no significant difference between the wild type and mutant during exposure to oxygen.

## MATERIALS AND METHODS

**Strains and growth conditions.** *B. fragilis* 638R (rifampin resistant) (30) was grown anaerobically in brain heart infusion broth supplemented with hemin, cysteine, and $NaHCO_3$ (BHIS) for routine cultures and genetic procedures (39). For radiolabelling experiments, cultures were grown in the chemically defined medium of Varel and Bryant (45) with vitamin $B_{12}$ replacing L-methionine. Anaerobic cultures were incubated at 37°C in an anaerobic chamber with an atmosphere of 80% $N_2$–10% $CO_2$–10% $H_2$. For viable-cell count assays, bacteria were grown in BHIS broth to mid-log and late-log/early-stationary growth. Cultures were diluted in BHI broth, plated out on BHIS agar containing 50 μg of gentamicin per ml, and incubated anaerobically for 3 to 5 days. Induction of the peroxide stress response with a sublethal concentration of hydrogen peroxide was carried out as follows. Cultures were treated with 50 μM $H_2O_2$ for 15 min with a second addition of 50 μM $H_2O_2$ for 15 min. Then cultures were split in several 10-ml aliquots and treated with either 1, 2.5, 5, or 10 mM $H_2O_2$ for 15 min. 2,2′-bipyridyl (150 μM) was added as indicated. Bovine liver catalase (10 μg/ml) was added to the dilution broth.

**Radiolabelling procedures.** Bacteria were grown to either late log phase (optical density at 550 nm = 0.7 to 0.8 [ca. $7 \times 10^8$ cells per ml) or mid-log phase (optical density = 0.3 [ca. $1.3 \times 10^8$ cells per ml]) as indicated. Cultures were divided into two equal volumes. One half was placed on a rotatory incubator at 37°C and shaken at 250 rpm in air, and the other half was kept in the anaerobic chamber. Radiolabelling was carried out by exposing bacteria to air for 15 min, and then 5 μCi of L-[$^{35}$S]methionine (Tran-$^{35}$S-label; ICN, Inc.) per ml (specific activity of 1,027 to 1,132 Ci/mmol) was added to both aerobic and anaerobic cultures. Labelling was carried out for 45 min and stopped by the addition of 0.5 mM cold L-methionine, 0.5 mM L-cysteine, and 100 μg of chloramphenicol per ml. Bacterial cells were pelleted and washed twice in 50 mM Tris-HCl (pH 8.0)–0.15 M NaCl, and crude cell extracts were obtained either by cell disruption in a French press or by boiling in SDS lysis buffer (29). The treatment of cultures with paraquat was carried out as described above for oxygen, except that after 5 min of culture aeration, 50 μM paraquat was added to both anaerobic and aerobic cultures. Radiolabelling in the presence of hydrogen peroxide was performed anaerobically by the addition of 50 μM $H_2O_2$ 15 min before the addition of labelled amino acid. Second additions of paraquat and peroxide were added after the addition of radiolabelled material. Novobiocin (200 μg/ml) or NaCl (0.5 M) was added to cultures as indicated.

**PAGE.** Nondenaturing PAGE and SDS-PAGE were carried out by the method of Laemmli (18) with a 7.5 to 20% exponential acrylamide gradient. After electrophoresis, the gels were stained by Coomassie blue R250, vacuum dried, and autoradiographed. Molecular weight standards were run in parallel.

The method of O'Farrell (27) was used for two-dimensional (2-D) gel electrophoresis. The first-dimension isoelectric focusing was performed with 2% ampholites (1.6% [pH 5 to 8] and 0.4% [pH 3 to 10]). Gels were loaded with from $5 \times 10^5$ to $10^6$ cpm. 2-D gel protein standards (Bio-Rad) were used as internal markers. The second-dimension SDS-PAGE was carried out as described above.

**Construction of the catalase-deficient mutant.** A catalase-deficient *B. fragilis* mutant was constructed by using an allelic exchange system to transfer a deleted catalase gene into the chromosome of *B. fragilis* 638R. The tetracycline gene, *tetQ*, was used as a selectable marker, replacing an internal fragment of *katB*. Briefly, pFD567 (33), which contains the entire *katB* gene, was digested with *Eco*RV and *Msc*I to remove a 639-bp internal fragment from *katB*. The digested plasmid was purified and then ligated to a 2.6-kb blunt-ended *Sst*I DNA fragment containing *tetQ*. Then this intermediate plasmid was partially digested with

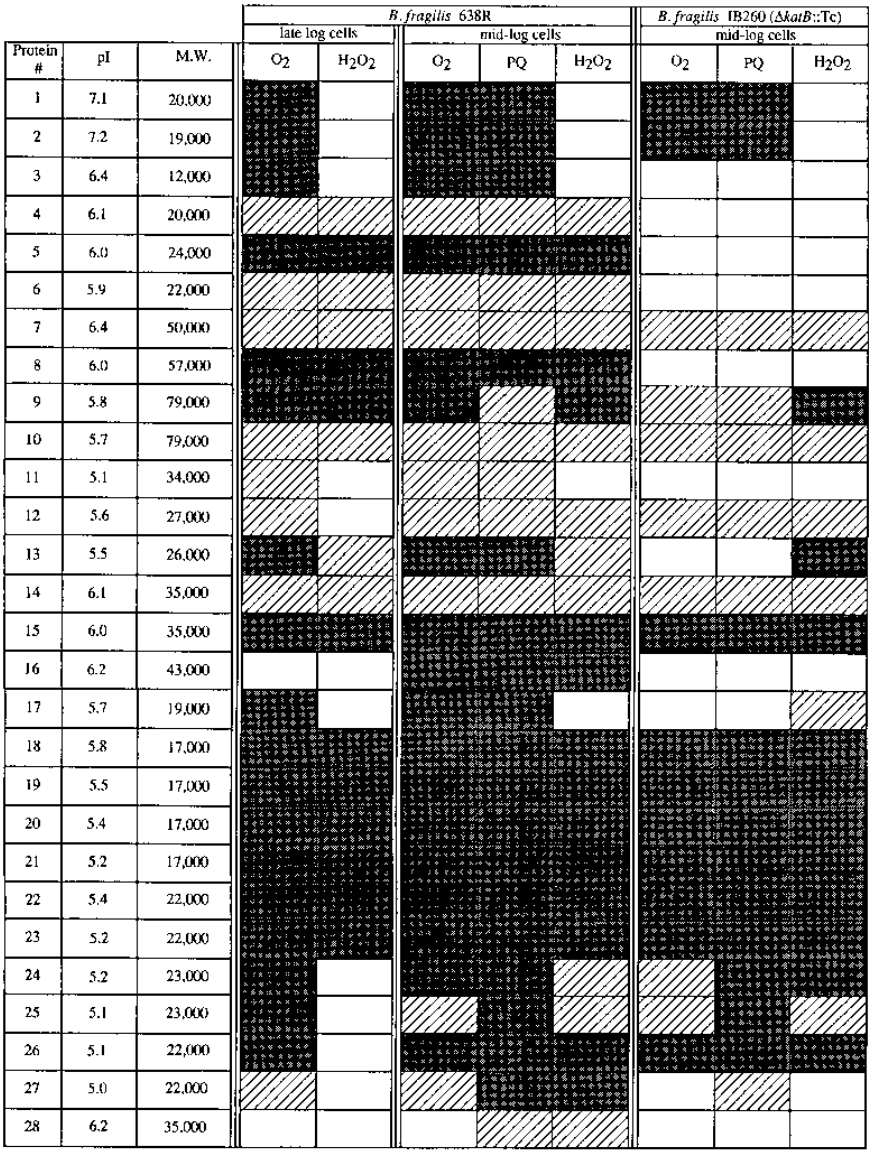


FIG. 2. Schematic diagram showing the oxidative stress response proteins observed under the conditions indicated. Protein numbers are based on comparisons with Fig. 1B. Gray boxes indicate the presence of proteins induced after oxidative stress. Hatched boxes indicate proteins that increased in level after oxidative stress. PQ, paraquat; M.W., molecular weight.

*Eco*RI, after which a 4.5-kb DNA fragment was isolated and ligated into the *Eco*RI site of the suicide vector pFD516 (40). Finally, a 3.8-kb *Bam*HI fragment containing the *sacB*/neomycin resistance cassette gene (2) was cloned into the *Bam*HI site of the plasmid to produce the final product, pFD611.

Plasmid pFD611 was mobilized from *Escherichia coli* DH5α by triparental mating into *B. fragilis* 638R using standard filter mating protocols (37). Exconjugants were selected on BHIS agar containing 20 μg of rifampin per ml, 25 μg of gentamicin per ml, 5 μg of tetracycline per ml, and 5% sucrose. Large and small recombinant colonies were observed, and these were cultured in the presence of either tetracycline or clindamycin to identify recombinants that were tetracycline resistant and clindamycin sensitive.

To complement the catalase activity in the mutant strain IB260, a 7.5-kb chromosomal DNA fragment containing the native catalase gene, *katB*, from *B. fragilis* 638R (33) (unpublished data) was cloned into the multicopy shuttle vector pFD288 (40). The construct, pFD630, was mobilized into IB260 by triparental mating, and exconjugants were selected on BHIS agar containing 20 μg of rifampin per ml, 25 μg of gentamicin per ml, and 5 μg of clindamycin per ml. The presence of catalase activity in clones was verified by enzymatic assay as described previously (33), and one representative clone was designated IB260(pFD630).

## RESULTS

**The oxidative stress response.** Several lines of evidence have suggested that aerotolerance in *B. fragilis* is conferred by an inducible, adaptative response to an oxidative insult (9, 31, 33, 34, 36, 38, 42). To investigate in more detail the oxidative stress response in *B. fragilis*, we used 2-D PAGE to analyze the protein profile induced by oxidative stress. Figure 1 presents the protein patterns of *B. fragilis* after oxidative stress induced by oxygen. Proteins indicated on autoradiographs from oxidatively stressed cells were identified by visual inspection, noting either the appearance of newly induced proteins or increases in the levels of existing proteins relative to the anaerobic control (Fig. 1A). Each of the new proteins was given an identification number (Fig. 2). This number was based on the standard 2-D protein map of an oxygen-exposed culture of *B. fragilis* (Fig.

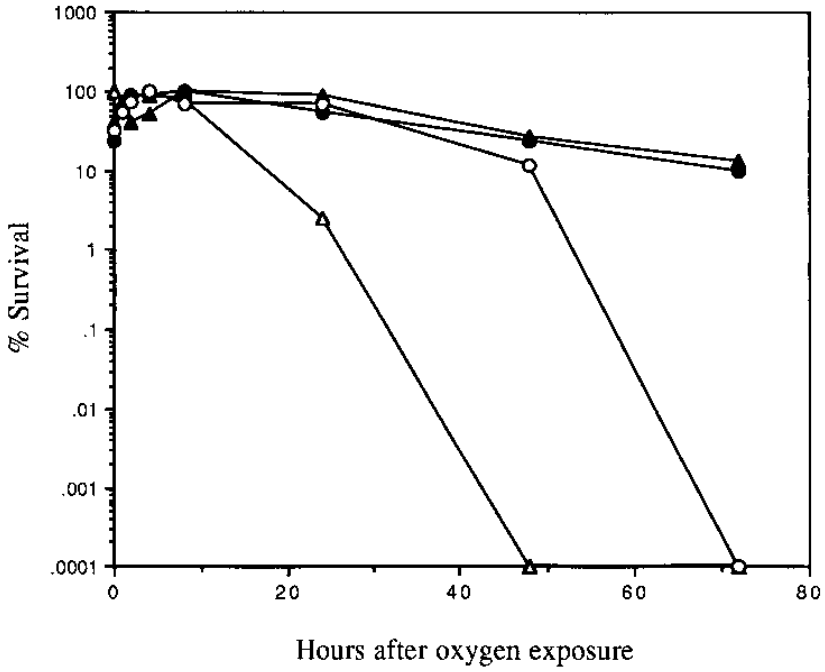


FIG. 3. Survival of *B. fragilis* 638R after exposure to oxygen in either the presence (△) or absence (○) of 25 μg of chloramphenicol per ml; anaerobic cultures not exposed to oxygen (▲ and ●, respectively).

1B), and a protein had to appear in at least two independent trials to be included in Fig. 2.

The *B. fragilis* protein profile was significantly altered after a shift from anaerobic to aerobic conditions or to aerobic conditions with paraquat and by the addition of $H_2O_2$ to anaerobic cultures. Approximately 28 new proteins synthesized in response to oxidative conditions were detected by 2-D gel electrophoresis. Among these proteins, 26 were induced by oxygen and 16 were induced by hydrogen peroxide in late-log-phase cells. In mid-log-phase cells, 27 proteins were induced by oxygen, 28 were induced by paraquat in the presence of oxygen, and 23 were induced by hydrogen peroxide. These proteins exhibited a broad range of pI values and molecular weights. They were found at a pI range from 5.1 to 7.2 and a molecular weight range from 12,000 to 79,000 (Fig. 1 and 2). The pattern displayed in Fig. 2 reveals a considerable overlap among the proteins induced under the oxidative conditions used in this study. Eleven of the oxidative response proteins (proteins 17 to 27 [Fig. 2]) showed consistent increases in their levels under the conditions used in this study. Interestingly, these proteins are closely related, with pI values from 5.1 to 5.8 and molecular weights from 17,000 to 23,000. This set of small, acidic proteins is apparently a major component of the general inducible oxidative response. We also observed that the shift of cultures from anaerobic to aerobic conditions caused the disappearance of many proteins while others remained unchanged. Although the regulation of such proteins may be involved in the physiological adaptation to oxidative stress, they were not considered further in this study.

The close interrelationship between oxygen and $E_h$ suggested that the oxidative stress response could be induced by high-redox conditions. This was tested by artificially raising the $E_h$ of anaerobic cultures by the addition of potassium ferricyanide to 100 μM (28). The anaerobic 2-D gel protein profile was not altered at this concentration of oxidizing agent (data not shown), suggesting that the oxidative stress response was not induced by an increase in the $E_h$ of the culture. To find out whether the oxidative stress response overlaps with other stress conditions, bacterial cultures were exposed to 200 μg of novobiocin per ml or 0.5 M NaCl. Except for protein 3, which was induced after the addition of novobiocin (data not shown), neither stress condition caused induction of the small, acidic proteins or other major oxidative stress proteins shown in Fig. 2.

If the oxygen stress response is an important physiological adaptation for protection against oxygen toxicity, then inhibition of this response should lead to impaired survival upon oxygen exposure. To test this idea, anaerobic *B. fragilis* cultures were exposed to oxygen in the presence or absence of 25 μg of chloramphenicol per ml and viable-cell counts were performed (Fig. 3). Under anaerobic conditions, the inhibition of protein synthesis did not significantly affect bacterial survival over 72 h when compared with the no-antibiotic anaerobic control. In contrast, aerated cultures containing chloramphenicol lost approximately 99% of viable cells in the first 24 h and an approximately $10^5$-fold decrease occurred by 48 h. Cell viability in the aerobic culture control without chloramphenicol did not decrease significantly until 48 h. After that time, there was a rapid loss of viability, confirming the oxygen toxicity to *B. fragilis.*

**Role of catalase in the oxidative stress response.** Considering the importance of catalase in the oxidative stress response of facultative anaerobes, it was of interest to determine the role of catalase in the survival of *B. fragilis* under aerobic or oxidative stress conditions. For these experiments, a *B. fragilis katB* mutant was constructed (Fig. 4A) as described in Materials and Methods. When pFD611 was transferred into *B. fragilis* 638R, large and small transconjugant colonies were obtained on tetracycline-sucrose medium. The large colonies were clindamycin sensitive, suggesting that the suicide vector did not remain integrated in the chromosome and that a double-crossover allelic recombination occurred. To confirm the structure of the catalase mutant, a Southern blot with *Eco*RI-

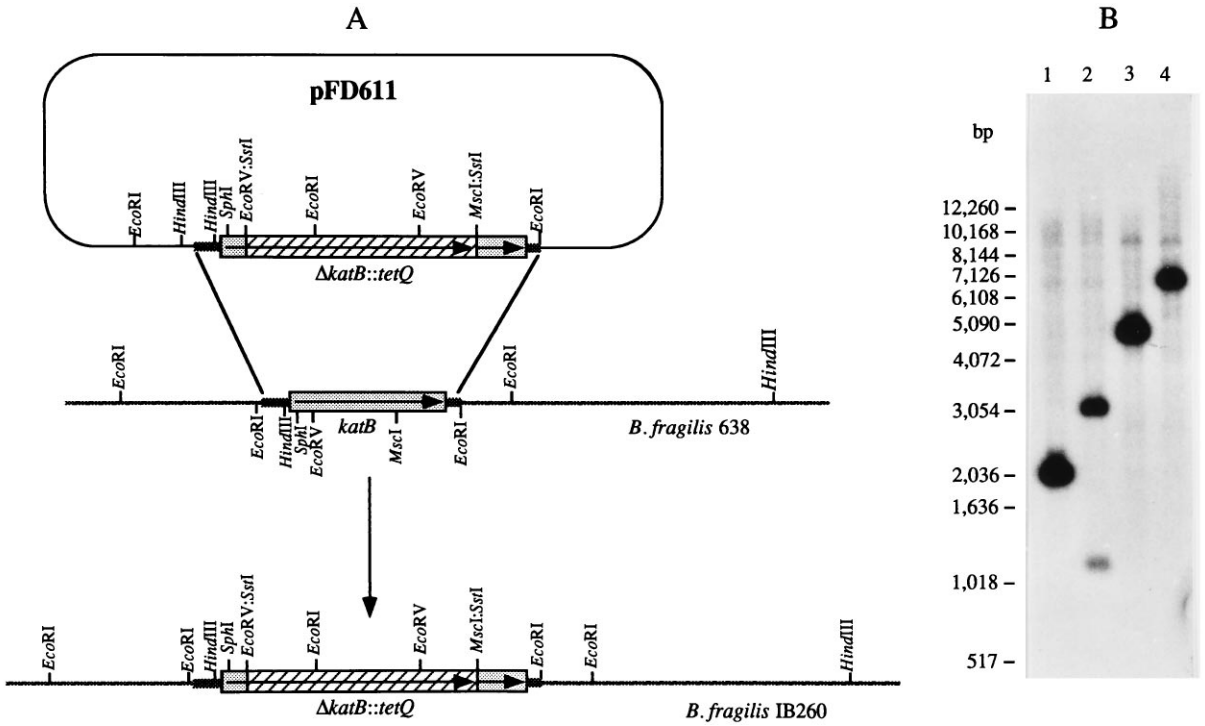


FIG. 4. Southern hybridization analysis and schematic diagram of the recombinant plasmid pFD611 and *B. fragilis* 638R allelic exchange mutagenesis. (A) Simplified double-crossover recombination diagram, showing the transfer and integration of the mutant *katB* gene from pFD611 into the homologous region of the *B. fragilis* chromosome. Partial restriction maps of the chromosome regions of the native *katB* in parent strain 638R and the mutant *katB* gene in IB260 are shown. (B) Autoradiograph of Southern blot analysis. Lanes 1 and 3, *B. fragilis* 638R chromosomal DNA digested with *Eco*RI and *Hin*dIII, respectively; lanes 2 and 4, *B. fragilis* IB260 chromosomal DNA digested with *Eco*RI and *Hin*dIII, respectively. The 2.0-kb *Eco*RI DNA fragment containing the native *katB* gene was used as a probe.

and *Hin*dIII-restricted chromosomal DNA was hybridized to a *katB*-specific gene probe (Fig. 4B). The autoradiograph of Southern blot analysis revealed that in strain IB260, the native catalase gene, *katB*, was replaced by the Δ*katB*::Tc gene. The evidence for this was best seen in the *Hin*dIII-digested samples (Fig. 4B, lanes 3 and 4), in which the hybridizing band showed an increase from approximately 4.8 to 7 kb, as predicted from the restriction map of pFD611. In addition, a new *Eco*RI site from the *tetQ* gene was introduced into the fragment (Fig. 4B, lane 2).

Two lines of evidence indicated that the *katB* mutant, IB260, was defective for catalase. First, the position of the catalase enzyme on 2-D PAGE gels (protein 8 [Fig. 1B and 2]) was identified by comparing the location of purified enzyme (33) on 2-D gels containing radiolabelled crude cell extracts. The catalase protein was no longer observed on 2-D gels of mutant strain IB260 after oxidative stress (Fig. 5). Second, catalase activity was no longer detected in whole cells or cell extracts of IB260 (data not shown).

The role of KatB in the oxidative stress response was examined first by viable-cell counts. When *B. fragilis* IB260 was exposed to aerobic conditions, viable-cell counts revealed that the absence of catalase enzyme did not significantly affect the survival of the mutant strain compared with that of the parent strain culture control (data not shown). 2-D PAGE analysis of IB260 cultures exposed to oxygen indicated that except for the absence of KatB, there was very little difference in the responses of the mutant and wild-type strains during exposure to oxygen (Fig. 5). Most of the stress response proteins found in the parent strain were also present in the mutant IB260; the results from multiple gels are summarized in Fig. 2. The results shown in Fig. 5 demonstrate that in the mutant (as with the parental strain), there was little difference among the treatments used in their ability to induce the oxidative stress response. Nearly identical protein profiles were observed for oxygen-, oxygen-plus-paraquat-, and hydrogen peroxide-treated cells (Fig. 2 and 5).

Catalase has been shown to be an important enzyme for the survival of facultative organisms exposed to hydrogen peroxide. In order to test the effects of hydrogen peroxide on the survival of *B. fragilis*, special precautions were needed because production of the hydroxyl radical may occur after the addition of $H_2O_2$ due to the presence of free soluble ferrous iron under anaerobic conditions (13, 16). Therefore, the ferrous iron chelator bipyridyl was added to cultures just prior to the addition of hydrogen peroxide (16). In the presence of bipyridyl, a significant difference between *B. fragilis* 638R and *B. fragilis* IB260 was observed (Fig. 6). Pretreatment of the parent strain with a sublethal concentration of hydrogen peroxide protected bacterial cells against at least 10 mM $H_2O_2$, with only a minimal decrease in cell viability. In contrast, the catalase mutant under the same conditions lost approximately 2 log units in cell viability up to 5 mM $H_2O_2$ and nearly all cell viability at 10 mM $H_2O_2$. On the other hand, without induction of the peroxide response, neither the mutant nor parental strain was protected against high concentrations of hydrogen peroxide (Fig. 6). The importance of catalase for resistance to exogenous hydrogen peroxide was confirmed when the catalase gene, *katB*, was restored in the mutant IB260 by plasmid pFD630. The complementation of catalase abolished the lethal effect of a high concentration of hydrogen peroxide in the presence of bipyridyl (Fig. 7A).

Evidence that the inducible peroxide stress response is required for protection against $H_2O_2$ was obtained from exper-

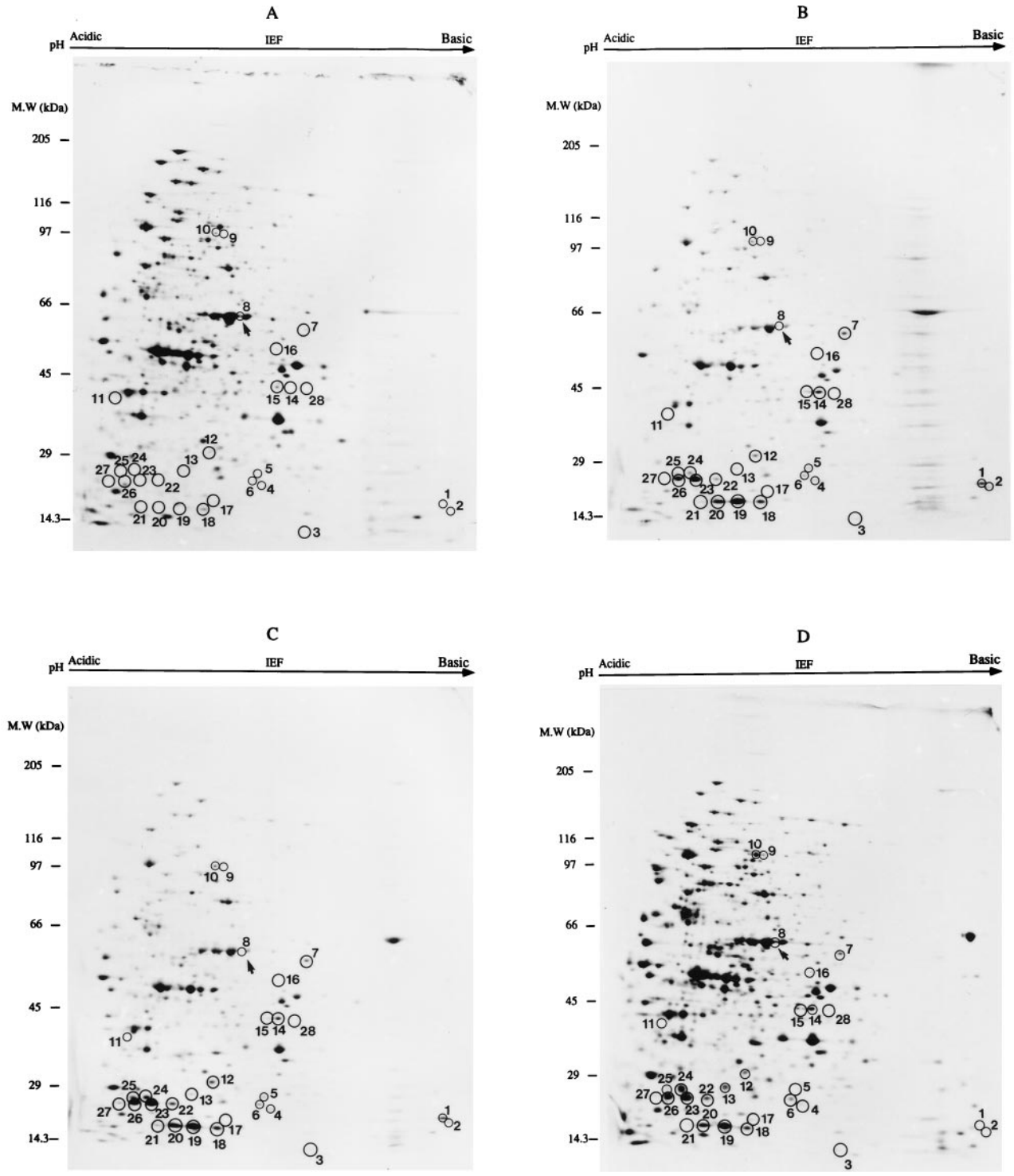


FIG. 5. Autoradiographs of 2-D electrophoresis of mid-log-phase cells of the catalase-deficient mutant *B. fragilis* IB260. (A) Anaerobic culture; (B) culture exposed to oxygen; (C) culture exposed to oxygen in the presence of paraquat (50 μM); (D) culture exposed to hydrogen peroxide (50 μM). In all panels, note that the KatB protein band is no longer observed; its predicted position is indicated by an arrow. M.W, molecular weight; IEF, isoelectric focusing.

iments in which chloramphenicol was added either before or after treatment of the anaerobic culture with a nonlethal dose of hydrogen peroxide. The results in Fig. 7B show the survival of these cultures in increasing concentrations of $H_2O_2$. The addition of chloramphenicol after induction with $H_2O_2$ had little effect on the survival kinetics, and cells were nearly fully protected against at least 10 mM $H_2O_2$. On the other hand, the inhibition of protein synthesis before induction of the peroxide response limited the ability of *B. fragilis* 638R to survive after further treatment with $H_2O_2$. It should be pointed out that the

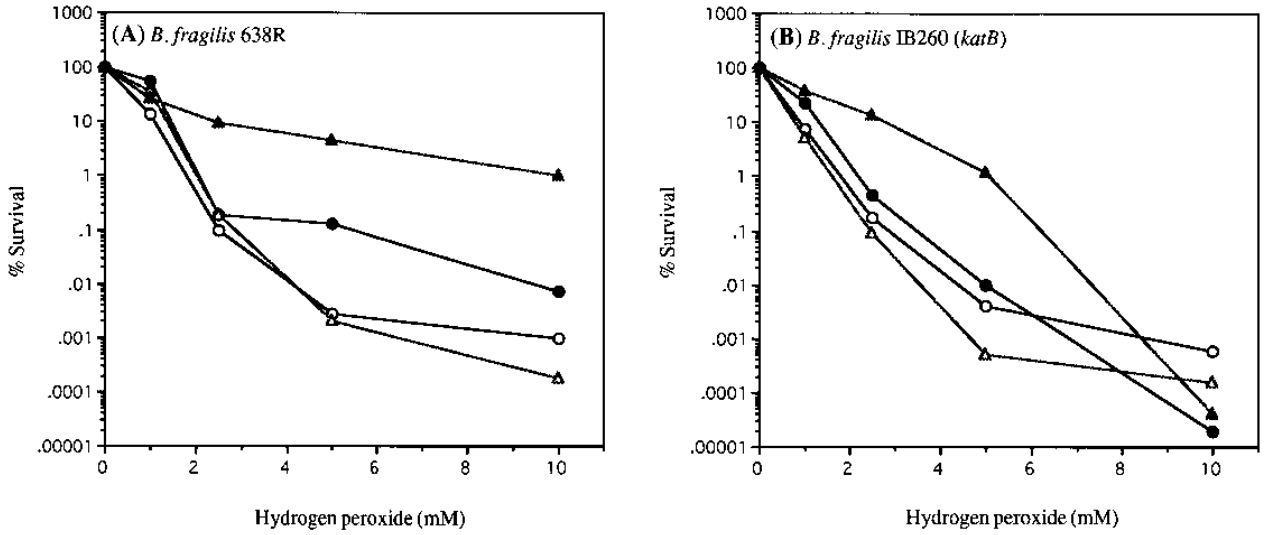


FIG. 6. Survival of mid-log-phase cells of *B. fragilis* 638R (A) and *B. fragilis* IB260 (B) after the addition of hydrogen peroxide for 15 min. ▲, pretreatment with 50 μM hydrogen peroxide in the presence of 150 μM bipyridyl; △, presence of 150 μM bipyridyl and no pretreatment with hydrogen peroxide; ●, pretreatment with 50 μM hydrogen peroxide and no addition of bipyridyl; ○, no pretreatment with hydrogen peroxide and no addition of bipyridyl.

inhibition seen in cultures treated prior to induction was somewhat greater than that observed for the *katB* mutant.

## DISCUSSION

The majority of obligate anaerobic bacteria do not survive for more than a few hours of oxygen exposure, but *B. fragilis* can survive for several days in the presence of air. We reasoned that survival would require specific genetic and phenotypic adaptations to the aerobic environment. The results presented here show that *B. fragilis* synthesizes a new set of proteins after oxidative stress. When cultures were shifted from anaerobic to aerobic conditions (with or without paraquat) or when $H_2O_2$ was added to cultures, 24 to 28 new proteins were induced, as detected by 2-D PAGE. With a few exceptions, most of the proteins listed in Fig. 2 show overlapping induction, indicating that the synthesis of these proteins may be due to a common response to oxidative stress. The importance of this response was shown when protein synthesis was inhibited at the time of culture aeration. Under these conditions, the survival of *B. fragilis* in the presence of oxygen was severely limited (Fig. 3). Addition of the oxidant potassium ferricyanide did not affect the protein profile of *B. fragilis* in the absence of oxygen, confirming that the response to oxidative stress may occur because of the presence of oxygen or oxygen derivative species rather than a high oxidation reduction state of the medium (28). Moreover, other stress conditions, such as osmotic shock and the inhibition of nucleic acid synthesis with novobiocin, did not cause the appearance of the major induced oxidative stress proteins. The coordinate expression (induction) of >25 new proteins provides strong evidence that a global mechanism regulates the oxidative stress response in *B. fragilis.*

The response of *B. fragilis* to hydrogen peroxide exposure was nearly identical to that seen with molecular oxygen or with

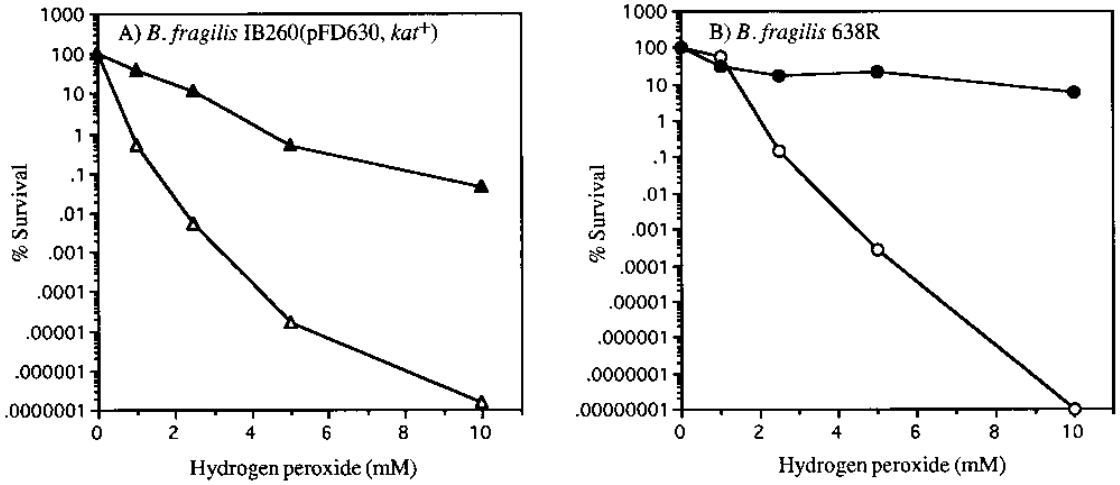


FIG. 7. Survival of mid-log-phase cells of *B. fragilis* IB260(pFD630) (*katB*$^+$) (A) and *B. fragilis* 638R (B) after the addition of hydrogen peroxide for 15 min in the presence of 150 μM bipyridyl. ▲, pretreatment with 50 μM hydrogen peroxide; △, no treatment; ●, 25 μg of chloramphenicol per ml was added after pretreatment with 50 μM hydrogen peroxide; ○, 25 μg of chloramphenicol per ml was added before pretreatment with 50 μM hydrogen peroxide.

oxygen plus paraquat (Fig. 2). Similarly, Schumann et al. (36) observed that three of four hydrogen peroxide-induced proteins seen by SDS-PAGE were common to those seen upon oxygen exposure. In addition, this $H_2O_2$-induced response was required to protect cells from subsequent higher doses of $H_2O_2$ (Fig. 6 and 7B). Overlapping responses appear to be a common strategy employed by a number of organisms, although the extent of the overlap of induced proteins seen with *B. fragilis* is rare. For example, in *E. coli*, only 3 of 14 paraquat-induced proteins were seen in cells stressed by $H_2O_2$ (46) and hydrogen peroxide-inducible proteins in *Salmonella typhimurium* were shown to be largely different from those proteins induced by aerobiosis (23). It is known that two different regulators, *soxRS* and *oxyR*, control the superoxide and peroxide responses, respectively (8, 43, 44).

The significance of the extensive overlap in *B. fragilis* oxidative stress responses is not known at present because there is not enough information regarding the specific physiological requirements for protection against oxygen toxicity. In fact, of the >25 peptides observed on 2-D gels of oxygen-stressed cells, only catalase has been unequivocally identified (compare Fig. 1 and 5). However, it is known that SOD activity is also induced when *B. fragilis* is exposed to oxygen (31). Both of these enzymes are likely to play a role in the protection of *B. fragilis* from ROS, and the lack of these enzymes in anaerobic bacteria was once thought to be the underlying cause of oxygen toxicity in anaerobes (24). However, on the basis of the results shown here as well as in previous studies, it would be an oversimplification to suggest that these two enzymes are sufficient for oxygen protection. On the other hand, the viability of an *sod* mutant of the anaerobe *Porphyromonas gingivalis* was greatly decreased in the presence of oxygen, suggesting that SOD is essential for this anaerobe (26).

As a first step toward understanding the role of oxygen-induced proteins in the survival of *B. fragilis*, a *katB* mutant was constructed and tested for aerotolerance and sensitivity to $H_2O_2$. The mutant was constructed by an allelic gene exchange method which has not been demonstrated previously with *Bacteroides* species and should prove to be useful for other mutant constructions of *B. fragilis*. When the *katB* mutant was exposed to oxygen, there was very little difference in survival compared with that of the parent strain. The lack of KatB activity did seem to have some effect on survival under anaerobic conditions in the stationary phase of growth, but this was not pursued further in this study. However, a similar observation was made for an *E. coli katE* mutant which lacks the HPII catalase controlled by the stationary-phase regulator *rpoS* (*katF*) (22, 25).

In contrast to oxygen exposure, the resistance to exogenous hydrogen peroxide under anaerobic conditions was significantly affected by the presence of a functional catalase enzyme in cells that were induced by pretreatment with a nonlethal concentration of hydrogen peroxide (Fig. 6). These findings are in agreement with previous reports showing that catalase-deficient strains of *B. subtilis* and *E. coli* are sensitive to exogenous hydrogen peroxide when compared with their respective parental strains (3, 19). In these organisms there is more than one catalase, and these enzymes are often under different levels of control, being induced by exogenous $H_2O_2$ or during the stationary phase of growth (3, 20, 21). *B. fragilis* appears to possess only one catalase which is clearly induced by conditions of oxidative stress brought on by exposure to either oxygen or $H_2O_2$. In this regard, it was surprising that under the oxygen exposure conditions tested, catalase was not required for survival. There may be several explanations for this result. The oxygen-stressed cells may not have generated sufficient levels of $H_2O_2$ internally to damage cellular structures. Alternatively, other components of the oxidative stress response may have been able to compensate for the loss of catalase in IB260.

The addition of the ferrous iron chelator bipyridyl seemed to aid in the detection of the role catalase activity plays in protecting bacterial cells against exogenous hydrogen peroxide. Under anaerobic conditions, most of the iron present in culture media is found in the reduced form (13). Thus, it is likely that after the addition of hydrogen peroxide, the toxicity observed against *B. fragilis* by hydrogen peroxide may have been caused in large part by the formation of ferryl and/or hydroxyl radicals, which readily occurs through the Fenton reaction rather than a direct effect of hydrogen peroxide itself (16). When the free ferrous iron in the medium was scavenged by the presence of bipyridyl, a clear protection against hydrogen peroxide toxicity in the parent strain 638R was observed but the catalase-deficient strain IB260 was much more sensitive to $H_2O_2$. This finding is consistent with several lines of evidence indicating that iron chelators can prevent the generation of hydroxyl radicals in in vivo systems (14, 16). It should be mentioned here that the addition of iron chelators did not affect the survival of *B. fragilis* in the presence of molecular oxygen (data not shown).

On the basis of the studies described here, we believe that the oxidative stress proteins in *B. fragilis* alleviate oxygen toxicity and allow the organism to survive for extended periods in aerobic environments. Although *B. fragilis* is a minor component of the total *Bacteroides* species found in the human intestine, it outnumbers all other species as a cause of extraintestinal anaerobic human infections (1, 5). Thus, the ability of *B. fragilis* to synthesize new proteins after an anaerobic-aerobic shift may be a particular advantage, allowing for survival in oxygenated host tissues until adequate anaerobic conditions are established and the organism adapts to the new life-style required for growth outside of the intestine.

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