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# Superoxide Dismutase in *Bacteroides fragilis* and Related *Bacteroides* Species

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Superoxide dismutase (SOD) activity was demonstrated in cell-free extracts of *Bacteroides fragilis*, *Bacteroides vulgatus*, *Bacteroides distasonis*, *Bacteroides ovatus*, and *Bacteroides thetaiotaomicron*. The strains were grown under anaerobic conditions in Trypticase soy broth, and the specific activity of SOD in the extracts was, in most strains, higher than in cell-free extracts of *Escherichia coli* B grown under anaerobic conditions. Isoelectric focusing of the extracts in polyacrylamide gel demonstrated distinct forms of SOD in the different species.

Anaerobic bacteria will not, by definition, grow on the surface of a solid medium freely exposed to air (16). Among these bacteria there is, however, a spectrum of oxygen sensitivity ranging from those for which oxygen is bactericidal at very low concentrations to those that tolerate exposure to air and for which atmospheric oxygen is bacteriostatic rather than bactericidal (12, 20). Various mechanisms have been suggested for the lethal effect of molecular oxygen (7, 17, 20, 21), and it seems likely that the toxicity of oxygen is multifactorial.

Many bacteria that use oxygen as an electron acceptor produce $H_2O_2$ and/or the superoxide free radical, $O_2^-$, both of which have the potential for causing cell damage (6). Most of these bacteria have catalase or peroxidase to decompose $H_2O_2$ and superoxide dismutase (SOD) to eliminate $O_2^-$ (17). Catalase is found in some anaerobic bacteria (4, 10, 22), and it was suggested that anaerobic bacteria could not tolerate molecular oxygen because they lack SOD (17). However, this enzyme has recently also been found in some anaerobic bacteria (8, 14, 26), and it is now realized that SOD is just one of many factors that protect an organism from the lethal effect of oxygen.

The saccharolytic bacteroides are among the predominant organisms of the large intestine of humans (19) and are often found in serious polymicrobial infections (23). *Bacteroides fragilis* is more often found in these infections than is any other nonsporing anaerobe (18, 23). Some strains of *B. fragilis* will grow at an oxygen tension up to 8% on blood agar plates and can tolerate exposure to air for several days (12, 25). SOD activity in *B. fragilis* and *Bacteroides distasonis* has recently been demonstrated (5, 26), and it was suggested that SOD may be a virulence factor that allows pathogenic anaerobes to survive in oxygenated tissues until the proper reduced conditions are established for their growth. We now demonstrate the presence of distinct forms of SOD in various species of the genus *Bacteroides*.

## MATERIALS AND METHODS

**Microorganisms.** The following reference strains were used: *B. fragilis* NCTC 9343 and 10584, *Bacteroides thetaiotaomicron* NCTC 10582, *B. distasonis* ATCC 8503, *Bacteroides ovatus* ATCC 8483, and *Bacteroides vulgatus* ATCC 8482 and NCTC 10583. Clinical isolates of these species were kindly supplied by E. Falsen, University of Gothenburg, and C.-E. Nord, State Bacteriological Laboratory, Stockholm, Sweden. *Escherichia coli* B was provided by S. Nordmark, Department of Microbiology, University of Umeå. The strains were stored in anaerobic double-strength skim milk at −80°C.

**Preparation of cell-free extract.** The bacterial cells and cell-free extracts were protected from oxygen during various phases of this work by anaerobic procedures previously described (27). The strains were grown in 250 ml of Trypticase soy broth (BBL, Cockeysville, Md.) for 18 h at 37°C in an anaerobic glove box. The cells were harvested by centrifugation at 13,000 × *g* for 30 min at 4°C, washed twice with 0.04 M potassium phosphate buffer, pH 7.0, and suspended in 5 ml of the same buffer with 1 drop of polypropylene glycol 2025 and 10 mg of dithiothreitol. Then, 4 ml of glass beads (0.10 to 0.11 mm) were added, and the cells were disintegrated for 1 min in a homogenizer (type MSK, B. Braun, Melsungen, Germany) under $CO_2$ cooling. Cell debris was removed by centrifugation at 40,000 × *g* for 60 min at 4°C. The supernatant fluid was designated as the cell-free extract and was stored under anaerobic conditions at −80°C.

*E. coli* B was also grown in aerated culture, and the cell-free extract was prepared under aerobic conditions in the same way as under anaerobic condi-

tions, but no dithiothreitol was added to the cell suspension.

**Assay of SOD activity.** SOD activity was determined according to Marklund and Marklund (15). Before the assay, 1 ml of cell-free extract was dialyzed in the anaerobic box for 18 h against 250 ml of 0.04 M potassium phosphate buffer, pH 7.0. A portion of the dialyzed extract was exposed to room atmosphere for 10 min before the assay, because gases of the anaerobic atmosphere dissolved in the extract interferred with the assay. The anaerobic atmosphere was 5% carbon dioxide and 10% hydrogen in nitrogen.

**Isoelectric focusing of SOD.** Isoelectric focusing, pH 3.5 to 9.5, was carried out in plates (245 by 110 by 1 mm) of polyacrylamide gel (LKB Produkter AB, Stockholm). The polyacrylamide concentration in the plates was 5%, the degree of cross-linkage was 3%, and the ampholine concentration was 2.4% (wt/vol). The isoelectric focusing was performed in LKB 2117 Multiphor apparatus according to the instructions of the manufacturer. The gel plate was put on the cooling plate (10°C) of the apparatus, and the electrode strips were applied on the gel. Filter paper pieces (Whatman 3MM, 10 by 15 mm) were soaked in the dialyzed cell-free extracts and placed onto the gel a few millimeters from the cathode electrode strip. Samples of hemolyzed horse blood (diluted 1:10) and purified SOD (1 U/ml, Truett Laboratories, Dallas, Tex.) were also applied on the gel. For the electrofocusing, the recommended power settings were used, and the filter paper pieces were removed after 40 min. The electrofocusing was stopped after about 1.5 h when the hemoglobulins of the horse blood showed optimal resolution. The areas of the gel covered by the electrode strips were cut away, and the gel was soaked at 4°C for 10 min in 100 ml of 0.2 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 9.0. The gel was then put into a tray with 40 ml of 0.2 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, containing 40 mg of nitroblue tetrazolium and 20 mg of phenazine methosulfate and was exposed at 25°C to a 40-W daylight fluorescent tube at a distance of 10 cm. When distinct achromatic zones against a uniform blue background had appeared, the gel was rinsed for a few minutes in a solution containing 250 ml of ethanol, 80 ml of acetic acid, and 100 ml of glycerol per liter. The gel was then stored in a solution containing 80 ml of acetic acid and 100 ml of glycerol per liter. As a control, the cell-free extracts were heated for 10 min at 100°C. No distinct achromatic bands appeared in the gel after this treatment of the extracts.

**Cultural characteristics of the strains.** The cultural characteristics of the strains were tested as described by Holdeman and Moore (10).

**Other analytical methods.** The protein concentration of cell-free extract was measured by the method of Lowry et al. (13). Fermentation products were analyzed as previously described (3, 27).

**Determination of oxygen tolerance.** The strains were grown on the surface of brain heart infusion agar medium (10) for 3 days at 37°C in the anaerobic box. The plates were brought out of the box, and the colonies on the plates were exposed to air for 1 week at 25°C in the dark. The survival of the strains was determined by taking samples of the colonies on the plates after 1, 2, 4, and 7 days and inoculating blood agar plates (10), which were then immediately put into the anaerobic box and incubated for 7 days at 37°C.

## RESULTS

All the strains survived for 1 week as colonies on the surface of brain heart infusion agar medium when exposed to atmospheric oxygen. SOD activity was found in all the strains. In cell-free extracts of most strains, at least two distinct bands of SOD activity could be demonstrated in isoelectric focusing gel (Fig. 1).

Two reference strains of *B. fragilis*, NCTC 9343 and NCTC 10584, and six clinical isolates were phenotypically homogenous (Table 1). SOD of all these strains formed a characteristic pattern (lane b) in the isoelectric focusing gel (Fig. 1).

Two reference strains of *B. vulgatus*, ATCC 8482 and NCTC 10583, and two clinical isolates had the same phenotypic characteristics (Table 1) and the same pattern (lane c) of SOD in the isoelectric focusing gel (Fig. 1). One strain with the same phenotypic characteristics as these strains had a distinct pattern (lane d) of SOD (Fig. 1).

Only one strain (ATCC 8503) of *B. distasonis* was studied. SOD of this strain formed a characteristic pattern (lane e) in the isoelectric focusing gel (Fig. 1).

Two strains of *B. ovatus*, ATCC 8483 and one clinical isolate, had SOD that formed two distinct patterns (lanes f and g) in the isoelectric focusing gel (Fig. 1).

Among the strains of *B. thetaiotaomicron*, four patterns of SOD in isoelectric focusing gel were found (Fig. 1). One clinical isolate, EF 3532, which was phenotypically different from the other (Table 1), had a pattern of SOD similar to that of NCTC 10582 (Fig. 1).

## DISCUSSION

Although *B. fragilis* and the other *Bacteroides* species will not grow on the surface of a solid medium freely exposed to air, the effect of the atmospheric oxygen is bacteriostatic rather than bactericidal (12, 25). *B. fragilis* also has a high resistance to inactivation by hyperbaric oxygen (9). Catalase activity has been found in some strains of *Bacteroides* (4, 5, 10), and, together with the SOD activity (5, 26), this could be important for the protection of these organisms against the toxic effect of oxygen. SOD activity was demonstrated in all the strains in the present study, and the specific activity of

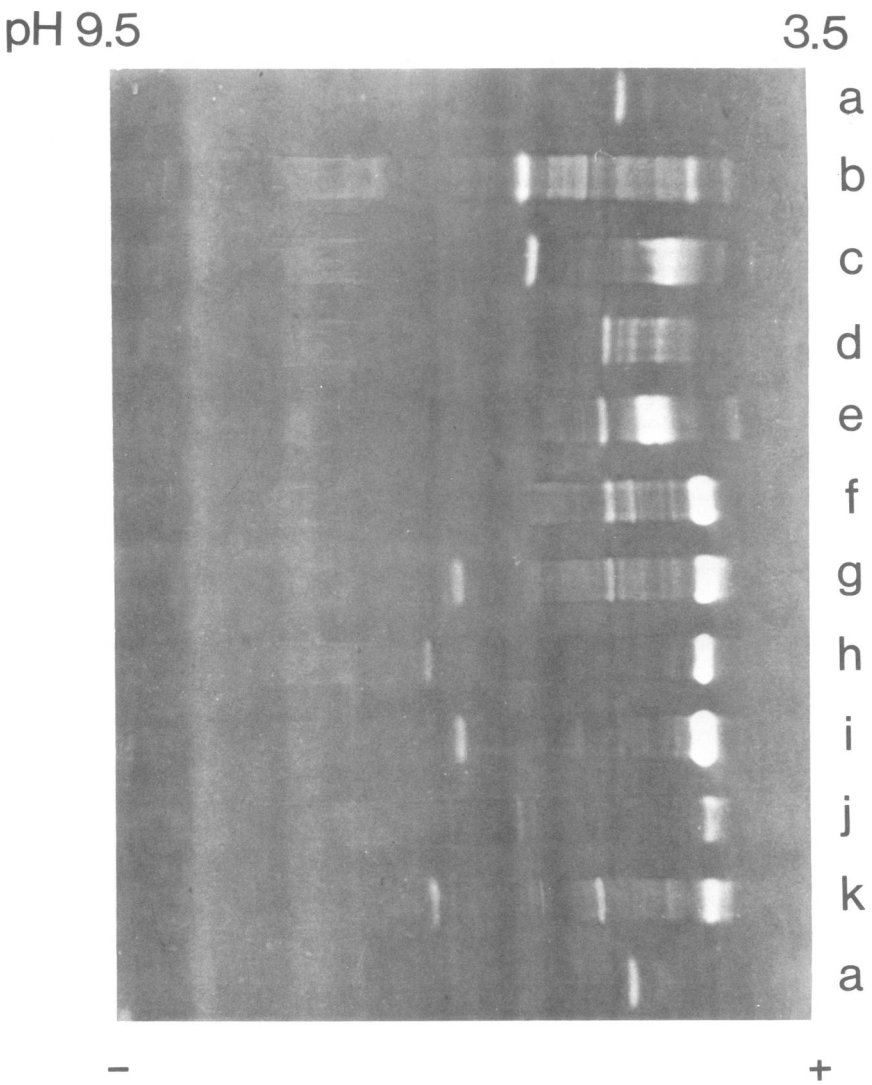


FIG. 1. *Patterns in isoelectric focusing polyacrylamide gel of SOD from various Bacteroides species. Cell-free extracts of the following strains were applied onto the gel (their specific activities, in units per milligram of protein, are given in parentheses) (b) B. fragilis NCTC 9343 (11), (c) B. vulgatus ATCC 8482 (18), (d) B. vulgatus EF 3932 (10), (e) B. distasonis ATCC 8503 (7), (f) B. ovatus ATCC 8483 (12), (g) B. ovatus EF 2097 (11), (h) B. thetaiotaomicron NCTC 10582 (5), (i) B. thetaiotaomicron SBL 1363 (7), (j) B. thetaiotaomicron SBL 1336 (5), (k) B. thetaiotaomicron EF 3532 (8). All these cell-free extracts were diluted 1:5 before they were applied onto the gel. Purified SOD from bovine erythrocytes (1 U/ml) was used as control (a). The specific activity of SOD in a cell-free extract of E. coli B grown anaerobically was 6 U/mg of protein and in aerobic culture it was 18 U/mg of protein. One unit of enzyme corresponds to 100 ng of bovine (Cu-Zn) SOD (15).*

the cell-free extracts was similar to that previously found in strains of *Bacteroides* grown under anaerobic conditions in complex media (5, 26). The specific activity of SOD has previously been found to vary with the composition of the growth medium (5, 26).

Most saccharolytic bacteroides of the human large intestine have been considered as strains of the species *B. fragilis*. Many characteristics of these strains are similar, but clusters of strains within this group with variable characteristics in indole production and acid production from particular sugars have been regarded as subspecies of *B. fragilis* (10). Nucleic acid homology studies, however, show that these subspecies have a high level of diversity in the arrangement of the deoxyribonucleic acid nucleotides (4, 11), and Beerens et al. (2) have

TABLE 1. *Cultural characteristics and SOD of 21 strains of Bacteroides*

| Cultural characteristic[a] | *B. fragilis* | | | *B. vulgatus* | | | *B. distasonis* | | | *B. ovatus* | | | *B. thetaiotaomicron* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strain | | SOD | Strain | | SOD | Strain | | SOD | Strain | | SOD | Strain | | SOD | Strain | | SOD |
| | NCTC | 9343 | b[b] | ATCC | 8482 | c[b] | ATCC | 8503 | e[b] | ATCC | 8483 | f[b] | NCTC | 10582 | h[b] | EF | 3532 | k[b] |
| | NCTC | 10582 | b | NCTC | 10583 | c | | | | EF | 2097 | g | SBL | 1363 | i | | | |
| | SBL | 1259 | b | SBL | 1262 | c | | | | | | | EF | 4289 | i | | | |
| | SBL | 1274 | b | SBL | 1310 | c | | | | | | | SBL | 1336 | j | | | |
| | EF | 2097 | b | EF | 3932 | d | | | | | | | | | | | | |
| | EF | 4091 | b | | | | | | | | | | | | | | | |
| | EF | 3996 | b | | | | | | | | | | | | | | | |
| | EF | 2101 | b | | | | | | | | | | | | | | | |
| Indole production | − | | | − | | | − | | | + | | | + | | | + | | |
| Acids from | | | | | | | | | | | | | | | | | | |
| Arabinose | − | | | + | | | − | | | + | | | + | | | − | | |
| Cellobiose | − | | | − | | | + | | | + | | | + | | | − | | |
| Mannitol | − | | | − | | | − | | | + | | | − | | | − | | |
| Rhamnose | − | | | + | | | − | | | + | | | + | | | + | | |
| Trehalose | − | | | − | | | + | | | + | | | + | | | + | | |
| Melibiose | + | | | + | | | − | | | + | | | + | | | − | | |
| Melezitose | − | | | − | | | − | | | + | | | ± | | | − | | |
| Ribose | − | | | ± | | | + | | | + | | | ± | | | − | | |
| Salicin | − | | | − | | | + | | | + | | | + | | | − | | |
| Sorbitol | − | | | − | | | − | | | ± | | | − | | | − | | |

[a] All strains produced succinic acid. They grew in the presence of 20% bile in peptone-yeast extract-glucose medium and hydrolyzed esculin and starch. They produced acids (pH < 5.75) from fructose, glucose, lactose, maltose, mannose, raffinose, starch, sucrose, and xylose. No strain produced acids from erythritol, inositol, dulcitol, glycerol, or sorbose.

[b] SOD pattern as shown in Fig. 1.

demonstrated that they are serologically distinct. Therefore, these subspecies have gained species rank (1, 4) and now constitute *B. fragilis*, *B. thetaiotaomicron*, *B. distasonis*, *B. ovatus*, and *B. vulgatus*. The present study demonstrates that reference strains of these species had SODs that formed distinct patterns in an isoelectric focusing gel. Many of the clinical isolates with cultural characteristics similar to the reference strains had SOD patterns in isoelectric focusing gel similar to those of the reference strains. These findings support the current speciation of the genus *Bacteroides*. In some of the clinical isolates, however, the SOD patterns were distinct from those of the reference strains. This heterogeneity among the strains was expected. Beerens et al. (2) demonstrated a number of serotypes within each of these species, and Johnson (11) found a number of deoxyribonucleic acid homology subgroups (strains having 80 to 100% homology to the reference strains). So far, no phenotypic traits have been found that delineate these homology subgroups. It is possible that the SOD pattern is a characteristic of each deoxyribonucleic acid homology subgroup and could be used for delineation. There appear to be some species-specific patterns of antibiotic susceptibility among *Bacteroides* species (24), and it is possible that a phenotypic delineation also of the homology subgroups could increase the reliability of predictions of antibiotic susceptibility. SOD has been considered as a virulence factor for anaerobic bacteria (26). From the result of the present study, it can be concluded that *B. fragilis* and related *Bacteroides* species have this factor.

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