Characterization of an Activity from the Strict Anaerobe

Roseburia cecicola That Degrades DNA when Exposed to Air

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Roseburia cecicola is an obligately anaerobic bacterium that is extremely sensitive to oxygen. Genomic DNA isolated from cells exposed to air for even a brief period (<5 min) is partially degraded, while DNA extracted from cells maintained in an anaerobic environment remains intact. Cells exposed to air for longer and longer periods yield DNA which is progressively degraded into fragments with decreasing sizes. Oxygen toxicity for this anaerobe appears to result, at least in part, from degradation of its genomic DNA. Cell lysates of the organism exhibited a similar ability to degrade exogenous sources of DNA when assayed in vitro under aerobic conditions. A substance that degrades both DNA and RNA when incubated aerobically was partially purified from such lysates. It has an approximate molecular weight of 2,800 and is unlikely to be a protein. It requires a reducing agent for activity and can be inhibited by catalase and peroxidase but not superoxide dismutase. The rate at which it degrades DNA in vitro can be enhanced by temperatures above 37°C or by oxygen at partial pressures above atmospheric pressure. These results suggest that this substance degrades nucleic acids by a mechanism involving oxygen radicals.

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**MATERIALS AND METHODS**

Culture conditions and oxygen toxicity. _R. cecicola_ GM was grown in prereduced, modified VTY medium in an atmosphere of 95% N₂ and 5% CO₂ (30). The cells were typically grown to a density of approximately 10⁶/ml unless otherwise indicated. Experiments to determine the toxicity of oxygen for _R. cecicola_ were conducted with cells grown anaerobically to the mid-exponential phase of growth (optical density at 600 nm, approximately 0.6). Laboratory air, N₂, or O₂ was then bubbled into cultures at a flow rate of approximately 0.5 liter/min for 1 min. The N₂ was passed over a heated copper column to remove residual O₂. After exposure to the various gases, the cultures were placed in an anaerobic chamber and the number of viable cells remaining in each was determined by plate counting. All manipulations throughout this study requiring anaerobiosis were performed in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) containing an atmosphere of 92% N₂, 5% CO₂, and 3% H₂.

DNA isolation. Chromosomal DNA was isolated from _R. cecicola_ in the anaerobic chamber as previously described (21), in accordance with a modified procedure of Marmur (20). Bacteriophage λ DNA was obtained from Sigma (St. Louis, Mo.), and plasmid (pBR322) DNA was purified from _Escherichia coli_ cell lysates by cesium chloride-ethidium bromide (EtBr) gradients. Chromosomal and λ DNAs were analyzed by electrophoresis through 0.4% agarose gels, while plasmid DNA was analyzed with 0.7% gels. DNA was visualized by staining with EtBr.

Purification of an activity that degrades DNA. Cells from 9-liter cultures of _R. cecicola_ were initially harvested, washed, and resuspended anaerobically in RSC buffer (50 mM NaCl, 15 mM sodium citrate, 10 mM cysteine, pH 7.4). Washed cells were then lysed by passage through a French pressure cell at 16,000 lb/in². The pressure cell and collection tube were flushed with a mixture of oxygen-free gas consisting of 95% (vol/vol) N₂ and 5% (vol/vol) CO₂ to minimize exposure of intact or lysed cells to air. Unlysed cells were removed by centrifugation at 12,000 × g for 15 min at 5°C.
The resulting supernatant solution (cell-free lysate) was returned to the anaerobic chamber and transferred into Quick-Seal centrifuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.). The sealed tubes were centrifuged at 150,000 × g for 2 h at 10°C to remove insoluble membrane fragments, and then the supernatant solution (crude extract) was collected. The crude extract was injected through a Sep-Pak Accell CM cation-exchange cartridge (Waters Associates, Inc., Milford, Mass.) previously equilibrated with rSSC buffer. Eluent exhibiting the ability to degrade DNA when exposed to air was then applied to a DEAE-Sepharose column (1.5 by 7 cm; Pharmacia LKB, Uppsala, Sweden) equilibrated with rSSC buffer. Fractions exhibiting activity were pooled and filtered through a stirred cell ultrafiltration unit (Pharmacia LKB) containing a membrane (Omega series) with a nominal molecular weight limit of 3,000. Filtrate exhibiting activity was collected and injected through a Sep-Pak C18 reverse-phase cartridge (Waters Associates) activated with methanol and equilibrated with rSSC buffer. Eluent exhibiting degrading activity, referred to as purified extract, was stored either aerobically at −80°C or in the anaerobic chamber at room temperature. All purification steps, excluding ultrafiltration, were performed in the anaerobic chamber. Ultrafiltration was conducted under an atmosphere of 100% CO2.

DNA degradation by purified extracts. Samples to be assayed for the ability to degrade DNA were transferred while in the anaerobic chamber into 2-ml microcentrifuge tubes. DNA (1 or pBR322) was added at a final concentration of approximately 0.01 mg/ml. The samples were then divided into two equal portions, both of which were incubated at 37°C for 1 h; one remained in the anaerobic chamber, while the other was incubated aerobically outside the chamber. Samples incubated aerobically were sparged with laboratory air for approximately 10 s every 15 min. Air flow was regulated with a flowmeter tube (Matheson, Montgomery, Pa.) to a rate of approximately 3 liters/min. In some experiments, samples of purified extract were incubated for time intervals ranging from 5 min to 24 h. Following sample incubation, 5 μl of loading dye (53% glycerol, 7% sodium dodecyl sulfate, 0.07% bromphenol blue) was added to each sample and DNA was analyzed by agarose gel electrophoresis. Samples of DNA, as well as all of the other materials used in these assays, were equilibrated in the anaerobic chamber for at least 24 h prior to use.

Molecular weight determination. The relative molecular weight (Mr) of the compound isolated in purified extracts which degraded DNA was calculated with a G-25 (fine) Sephadex (Pharmacia LKB) gel filtration column (1.5 by 80 cm). The standards used to calibrate the column were obtained from Sigma and included bovine insulin (Mr, 5,734), porcine adrenocorticotropic hormone (Mr, 4,567), bovine insulin β chain (Mr, 3,496), and vitamin B12 (Mr, 1,355). Fractions collected from the column were tested for the ability to degrade DNA as described above.

Dialysis of purified extracts. Samples of purified extract were transferred into Spectra/Por Molecularporous membranes (Spectrum Medical Industries, Inc., Los Angeles, Calif.) with a molecular weight cutoff of 1,000 and dialyzed in the anaerobic chamber against either a reduced Tris buffer (50 mM Tris [pH 7.4], 50 mM NaCl, 10 mM cysteine) or a reduced PO4 buffer (50 mM sodium phosphate [pH 7.4], 50 mM NaCl, 10 mM cysteine). The retentate from each experiment was subsequently tested for the ability to degrade DNA. In other experiments, purified extracts were dialyzed against SSC (0.05 M NaCl plus 0.015 M sodium citrate) buffer that did not contain cysteine. The resulting retentate was then assayed for the ability to degrade DNA with other reducing agents present.

Effect of incubation conditions on the ability of purified extracts to degrade DNA. The influence of oxygen, temperature, and pH on the ability of purified extracts to degrade λ DNA (0.01 mg/ml) was examined. Following the described treatment, samples were analyzed for the ability to degrade DNA by agarose gel electrophoresis as previously described.

(i) Oxygen. Samples of purified extract and DNA were transferred while in the anaerobic chamber to airtight glass vials and crimp sealed with a rubber septum-type stopper. Samples were subsequently removed from the chamber and incubated at 37°C for 1 h while being sparged every 15 min for 10 s with a gas mixture consisting of various relative concentrations (vol/vol) of O2 and oxygen-free N2.

(ii) Temperature. Samples of purified extract were incubated with DNA either anaerobically or aerobically for 1 h at either 4, 23, (room temperature), 37, 45, or 60°C. In other experiments, samples of purified extract were incubated at 100°C for 30 min, allowed to cool, and then assayed for the ability to degrade DNA.

(iii) pH. DNA was added to samples of purified extract, and the pH of the mixture was adjusted with autoclaved solutions of HCl or NaOH to approximately 4, 5, 6, 8, 9, or 10 while in the anaerobic chamber. The samples were then divided into two equal portions and removed from the anaerobic chamber. The pH of one of these samples was confirmed with a microcombination pH electrode (Micro-electrodes, Inc., Londonderry, N.H.), while the other was incubated aerobically at 37°C for 1 h.

Inhibition studies. Protease (protease K [specific activity, 20 U/mg of solid] or pronase E [specific activity, 5.8 U/mg of solid]) or various enzymes (superoxide dismutase [SOD], catalase, or peroxidase) involved in the removal of certain reactive oxygen species were tested for the ability to inhibit purified extracts from degrading DNA (0.01 mg of λ or pBR322 DNA per ml). SOD (bovine erythrocyte; specific activity, 3,600 U/mg of solid), catalase (bovine liver; specific activity, 44,000 U/mg of protein), or horseradish peroxidase (specific activity, 1,480 U/mg of solid) was added to samples of purified extract along with the DNA, while proteases were incubated anaerobically with samples of purified extract for 1 h at 37°C prior to the addition of DNA. The samples were then incubated either anaerobically or aerobically at 37°C for 1 h. All enzymes were reconstituted or, in the case of catalase, diluted in 50 mM phosphate buffer, pH 7.4, just prior to assay. Proteases were incubated at 37°C for 2 h prior to assay to remove any of the endogenous DNase activity frequently present in these preparations.

Enzyme assays. Cell-free lysates of R. cecicola were assayed for SOD, catalase, and peroxidase activities. Lysates were prepared as previously described, except that cells were washed and resuspended in 50 mM sodium phosphate buffer, pH 7.4, prior to disruption in the French press. The protein concentration of cell-free lysates was determined by the method of Bradford (3). SOD activity was assayed by the method of McCord and Fridovich (23), while catalase activity was measured by a method described by Beers and Sizer (2). A method described elsewhere (35) was used to assay cell extracts for peroxidase activity.
RESULTS

Oxygen toxicity. The toxicity of oxygen for *R. cecicola* was initially examined by exposing cells to oxygen and determining its effect on viability. Oxygenated environments are extremely toxic for *R. cecicola*, as indicated by the bactericidal effects observed when oxygen or air, but not nitrogen, was bubbled into an actively growing culture. Cell growth, as determined by monitoring the optical density at 600 nm (Fig. 1), was immediately inhibited when cultures were exposed to air or O₂ for only 1 min. Moreover, no viable cells were detected in these cultures following such treatment. Cell growth was not inhibited, however, in cultures exposed to oxygen-free N₂.

Reactive oxygen species, such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂), have been associated with oxygen toxicity in biological systems. We assayed *R. cecicola* cell lysates for certain enzymes involved in the removal of these species. No detectable levels of either SOD or catalase were observed in the cell lysates.

DNA degradation. Chromosomal DNA extracted from cells of *R. cecicola* exposed to air was partially degraded, while DNA extracted in an oxygen-free environment remained intact and of high molecular weight. DNA isolated from cells exposed to air for longer and longer periods was progressively fragmented into pieces with smaller and smaller sizes (Fig. 2). This phenomenon was observed whether cells exposed to air were in the exponential or stationary phase of growth. Chromosomal degradation could not be inhibited by incubating cells with antibiotics that inhibit transcription (100 μg of rifampin per ml) or translation (200 μg of chloramphenicol per ml) for 30 min prior to aerobic exposure. This phenomenon was not an artifact of the buffer used in the extraction procedure, since DNA was partially degraded when isolated aerobically with either rSSC or rSTE (100 mM NaCl, 10 mM Tris [pH 7.0], 1 mM EDTA, 10 mM cysteine) buffer. Similar results were obtained when the reducing agent (10 mM cysteine) was excluded from these buffers (data not shown).

Purification of DNA-degrading activity. A substance(s) that degrades DNA when incubated aerobically but not anaerobically was partially purified from cells of *R. cecicola*. The material did not bind to either a cationic (carboxymethyl) or a reverse-phase (C₁₈) adsorbent but was retained slightly by an anion-exchange adsorbent (DEAE). Its apparent molecular weight was estimated to be between 1,000 and 3,000 on the basis of ultracentrifugation and dialysis and subsequently confirmed to be 2,500 by gel filtration chromatography. Since the compound did not bind to any of the previously mentioned chromatographic matrices, attempts to purify it to homogeneity were unsuccessful. Although some impurities undoubtedly are present in these purified extracts, most of the cellular proteins have been removed. For example, protein concentrations in cell lysates varied between 50 and 150 mg/ml whereas the protein concentration of purified extracts was less than 0.01 mg/ml. The absorption spectra of different preparations of purified extract (data not shown) revealed no peaks between the wavelengths of 400 and 800 nm (visible region). A broad absorption peak extending from 220 to 320 nm and centered at approximately 250 nm was evident, however, in the UV region of the spectrum (200 to 400 nm).

DNA degradation by purified extracts. The material in the purified extracts was able to degrade exogenous DNAs from a variety of genomic sources (e.g., calf thymus and bacteriophages T₄, T₅, and λ) when incubated in air. Methylated and nonmethylated forms of λ DNA were degraded to the same extent. Extracts degraded DNA into smaller fragments as they were incubated aerobically for longer periods (Fig. 3). DNA was not degraded when incubated either anaerobically with purified extracts or aerobically with rSSC. Plasmid (pBR322) DNA was also degraded when incubated aerobically with purified extracts, although not all topological forms were degraded to the same extent (data not available).
shown). Covalently closed circular forms of plasmid DNA began to disappear following a brief (5-min) aerobic incubation with extracts, while open circular forms concomitantly became visible. In most experiments, most of the covalently closed circular forms were converted to open circular forms within 1 h. Further nicking of open circular DNA was indicated by the appearance (<15 min) of linearized plasmid. The open circular and linear forms of plasmid DNA were still present, however, after 9 h of aerobic incubation. Significant amounts of linearized plasmid did not appear to be degraded by purified extracts in these assays. Plasmid DNA was not degraded when incubated aerobically with rSSC or anaerobically with purified extracts.

**Purified extracts require a reducing agent for activity.** The ability of purified extracts to degrade DNA was dependent upon certain components of the rSSC buffer. Citrate was not required for activity, as indicated by the ability of the extracts to degrade DNA after extensive dialysis against either Tris or phosphate buffer. Samples of purified extract dialyzed against SSC without cysteine, however, were no longer able to degrade DNA when incubated aerobically. Dialyzed extracts regained the ability to degrade DNA when various reducing agents, such as cysteine, ascorbate, dithiothreitol, β-mercaptoethanol, or sodium sulfide, were added at 10 mM (Fig. 4). DNA was not degraded when incubated aerobically with rSSC to which similar concentrations of reducing agents were added. Ascorbate at 10 mM appeared to enhance the ability of the extracts to degrade DNA. When N-ethylmaleimide, an inactivator of sulfhydryl groups, was incubated with purified extracts along with various reducing agents, only extracts incubated with ascorbate were still able to degrade DNA (data not shown).

**Effects of air on purified extracts.** Exposure of purified extracts to air at 37°C for increasing periods affected their ability to degrade subsequently added DNA. Samples of purified extracts preincubated aerobically for 1 h and 2.5 h exhibited a diminished capacity to degrade λ DNA, while samples preincubated for 5 h were no longer able to degrade DNA (Fig. 5). Similar results were obtained with purified extracts regardless of whether they were assayed for activity immediately after preincubation in air (data not shown) or whether they were allowed to equilibrate in the anaerobic chamber after exposure to air prior to being assayed.

**Effects of oxygen, temperature, and pH on DNA degradation.**
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DNA (lane greater than the incubation Lane 1 when influenced by pH temperatures. nonspecific degrading edited degraded DNA (600°C), at degraded DNA pronase nor extracts degrade DNA DNA-degrading enzymes between 5.5 and 8.5 temperature temperature was decreased. Temperatures greater than 60°C were not assayed, since rSSC also exhibited nonspecific degrading of DNA when incubated at these temperatures.

(iii) pH. The ability of extracts to degrade DNA was influenced by the hydrogen ion concentration of the buffer. Extracts degraded DNA at the fastest rate when incubated at the highest temperature assayed (60°C), while visibly less DNA was degraded as the incubation temperature was decreased. Temperatures greater than 60°C were not assayed, since rSSC also exhibited nonspecific degrading of DNA when incubated at these temperatures.

Inhibition of DNA-degrading activity. Neither proteinase K nor pronase E inhibited the degradation of either plasmid (data not shown) or λ (Fig. 9) DNA by extracts. To ensure that the proteases remained active under the conditions assayed, 100 μg of bovine serum albumin was incubated at 37°C for 1 h with samples of either rSSC, purified extract, or 50 mM phosphate buffer (pH 7.4). Native-molecular-weight bovine serum albumin was no longer visible when these samples were subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that the proteases retained their activity in samples of purified extracts.

Enzymes involved in the removal of reactive oxygen species exhibited a variable capacity to inhibit the degradation of DNA by purified extracts. SOD at a concentration of 10 μg/ml (36 U/ml) did not inhibit the degradation of plasmid (data not shown) or λ (Fig. 9) DNA by extracts. Similar results were observed when 50 μg of SOD per ml was assayed (data not shown). Purified extracts incubated aerobically with either 10 μg of catalase (440 U/ml) or peroxidase

In Fig. 6, the EtBr-stained agarose gel of purified extracts incubated with λ DNA at 37°C for 1 h in an atmosphere consisting of the following concentrations (vol/vol) of O2: 0% (lane 2), 2.5% (lane 3), 5% (lane 4), 10% (lane 5), 20% (lane 6), 50% (lane 7), 100% (lane 8). Lane 1 contained purified extract incubated anaerobically with λ DNA for 1 h at 37°C.
precursors (9, 10, 13, 24) is DNA enzymatic cells of was attempted to reactive extremely therefore.

**DISCUSSION**

Brief exposure to oxygenated environments is lethal to *R. cecicola*. A manifestation of oxygen toxicity in this anaerobe is the nonspecific degradation of its chromosomal DNA that occurs immediately following exposure to air. The DNA degrades progressively as the cells are exposed to air for longer and longer periods by a mechanism that does not require de novo transcription or translation.

On the basis of the manner in which chromosomal DNA was degraded, we hypothesized that oxygen radicals were involved in this phenomenon. Reactive species generated initially during the reduction of molecular oxygen (O$_2^-$) and H$_2$O$_2$ react very slowly with DNA (6, 15, 33) and are therefore unlikely to be directly responsible for chromosomal degradation. The hydroxyl radical (OH$^-$), however, which is formed upon further reduction of these species is extremely reactive with most biological molecules, including nucleic acids (5, 15, 16, 29). Hydroxyl radicals degrade DNA in a manner that is independent of the sequence and thus similar to the degradation of chromosomal DNA observed in cells of *R. cecicola*. In addition, this anaerobe lacks specific enzymatic antioxidants (SOD and catalase) which would minimize the amount of OH$^-$ generated within a cell by lowering the steady-state concentrations of O$_2^-$ and H$_2$O$_2$ precursors (9, 10, 13, 24, 32).

To study further the phenomenon by which chromosomal DNA is degraded in cells of *R. cecicola* exposed to air, we attempted to isolate from cellular lysates the compound(s) responsible for this activity. We purified from cells an extract that degrades exogenous sources of DNA in vitro under aerobic conditions in a manner identical to that observed for chromosomal DNA in cells exposed to air. In addition, the manner in which these purified extracts degrade nucleic acids is consistent with the involvement of hydroxyl radicals. For example, hydroxyl radicals, like purified extracts, degrade nucleic acids in a manner that is not inhibited by increases in temperature or moderate changes in pH (26, 27, 33). Moreover, the selective manner in which certain topological forms of plasmid DNA are degraded by purified extracts is identical to that observed when plasmid DNA is incubated with compounds that generate OH$^-$ via a Fenton reaction (4, 28, 33). We have also found that purified extracts are capable of degrading exogenous sources of RNA under aerobic conditions (unpublished data). This finding is consistent with the role of hydroxyl radicals, since they have been previously reported to degrade RNA nonspecifically (14, 17, 26).

Hydroxyl radicals are thought to be generated in biological systems predominantly when H$_2$O$_2$ is reduced by transition metals (1, 12, 18, 19, 33). Although a number of transition metals can catalyze this reaction in vitro, the most abundant and therefore the most probable metal to catalyze the reaction in vivo is iron (Fenton reaction). A sustained flux of OH$^-$ can only be generated, however, when the reduced form of the transition metal is continually recycled (Fe$^{3+}$→Fe$^{2+}$). This continuous flux is thought to underlie the extensive damage (e.g., fragmented DNA) often associated with oxygen toxicity. A number of molecules have been proposed to be capable of reducing Fe$^{3+}$ in vivo, including various thiols, NAD(P)H, ascorbate, and -O$_2^-$ (1, 12, 25, 34). The compound isolated in extracts from *R. cecicola* may generate OH$^-$ via a similar mechanism. Its requirement for a reducing agent and the ability of enzymes which remove H$_2$O$_2$ to inhibit or reduce its activity are consistent with this hypothesis.

On the basis of the purification scheme used to isolate this activity, purified extracts are unlikely to be homogeneous preparations. Certain physical properties of the compound that mediates DNA degradation, however, can be inferred from this study. Its molecular weight is approximately 2,800, it does not appear to be a protein, it cannot be inactivated by heat, and it is stable when stored under conditions that minimize exposure to oxidizing environments. Furthermore, since activity was consistently detected in discrete fractions eluting from both the gel filtration and anion-exchange columns, we hypothesize that the ability to degrade DNA is mediated by a single compound in these extracts. If more than one compound is required for DNA-degrading activity, they appear to be either firmly bound to one another or so similar in charge and molecular weight that they cannot be separated by these types of chromatography. Further purification of this compound is necessary before this hypothesis can be confirmed.

The toxicity of molecular oxygen is not unique to strictly anaerobic organisms. In fact, hyperbaric oxygen can be deleterious to most biological systems (11, 13), with the physical damage being as diverse as the biological systems it affects. *R. cecicola* is the first obligate anaerobe, however, that has been shown to exhibit extensive degradation of its genomic DNA upon exposure to oxygen. A similar phenomenon has been observed in certain strict aerobes (*Treponema pallidum* and *Neisseria gonorrhoeae*) and facultative anaerobes (*E. coli* and *Salmonella typhimurium*) in which oxygen radicals were generated artificially within the cells through.
REFERENCES